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## AMYLASE VARIANTS

### FIELD OF THE INVENTION

The present invention relates to  $\alpha$ -amylase variants having improved properties relative to the parent enzyme (e.g. improved thermal and/or oxidation stability  
5 and/or reduced calcium ion dependency), and thereby improved washing and/or dishwashing (and/or textile desizing) performance. The invention also relates to DNA constructs encoding the variants, and to vectors and cells harbouring the DNA constructs. The invention further relates to methods of producing the amylase variants, and to detergent additives and detergent compositions  
10 comprising the amylase variants. Furthermore, the invention relates to the use of the amylase variants for textile desizing.

### BACKGROUND OF THE INVENTION

$\alpha$ -Amylase enzymes have been used industrially for a number of years and for a variety of different purposes, the most important of which are starch liquefaction,  
15 textile desizing, starch modification in the paper and pulp industry, and for brewing and baking. A further use of  $\alpha$ -amylases which is becoming increasingly important is the removal of starchy stains during washing or dishwashing.

In recent years attempts have been made to construct  $\alpha$ -amylase variants having improved properties with respect to specific uses such as starch liquefaction and  
20 textile desizing.

For instance, US 5,093,257 discloses chimeric  $\alpha$ -amylases comprising an N-terminal part of a *B. stearothermophilus*  $\alpha$ -amylase and a C-terminal part of a *B. licheniformis*  $\alpha$ -amylase. The chimeric  $\alpha$ -amylases are stated to have unique properties, such as a different thermostability, as compared to their parent  
25  $\alpha$ -amylase. However, all of the specifically described chimeric  $\alpha$ -amylases were

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shown to have a decreased enzymatic activity as compared to their parent  $\alpha$ -amylases.

EP 252 666 describes hybrid amylases of the general formula Q-R-L, in which Q is a N-terminal polypeptide residue of from 55 to 60 amino acid residues which is at least 75% homologous to the 57 N-terminal amino acid residues of a specified  $\alpha$ -amylase from *B. amyloliquefaciens*, R is a specified polypeptide, and L is a C-terminal polypeptide comprising from 390 to 400 amino acid residues which is at least 75% homologous to the 395 C-terminal amino acid residues of a specified *B. licheniformis*  $\alpha$ -amylase.

10 Suzuki et al. (1989) disclose chimeric  $\alpha$ -amylases, in which specified regions of a *B. amyloliquefaciens*  $\alpha$ -amylase have been substituted for the corresponding regions of a *B. licheniformis*  $\alpha$ -amylase. The chimeric  $\alpha$ -amylases were constructed with the purpose of identifying regions responsible for thermostability. Such regions were found to include amino acid residues 177-186 and  
15 amino acid residues 255-270 of the *B. amyloliquefaciens*  $\alpha$ -amylase. The alterations of amino acid residues in the chimeric  $\alpha$ -amylases did not seem to affect properties of the enzymes other than their thermostability.

WO 91/00353 discloses  $\alpha$ -amylase mutants which differ from their parent  $\alpha$ -amylase in at least one amino acid residue. The  $\alpha$ -amylase mutants disclosed  
20 in said patent application are stated to exhibit improved properties for application in the degradation of starch and/or textile desizing due to their amino acid substitutions. Some of the mutants exhibit improved stability, but no improvements in enzymatic activity were reported or indicated. The only mutants exemplified are prepared from a parent *B. licheniformis*  $\alpha$ -amylase and carry one  
25 of the following mutations: H133Y or H133Y + T149I. Another suggested mutation is A111T.

FR 2,676,456 discloses mutants of the *B. licheniformis*  $\alpha$ -amylase, in which an amino acid residue in the proximity of His 133 and/or an amino acid residue in the

proximity of Ala 209 have been replaced by a more hydrophobic amino acid residue. The resulting  $\alpha$ -amylase mutants are stated to have an improved thermostability and to be useful in the textile, paper, brewing and starch liquefaction industry.

5 EP 285 123 discloses a method of performing random mutagenesis of a nucleotide sequence. As an example of such sequence a nucleotide sequence encoding a *B. stearrowthermophilus*  $\alpha$ -amylase is mentioned. When mutated, an  $\alpha$ -amylase variant having improved activity at low pH values is obtained.

In none of the above references is it mentioned or even suggested that  $\alpha$ -amylase  
10 mutants may be constructed which have improved properties with respect to the detergent industry.

EP 525 610 relates to mutant enzymes having improved stability towards ionic tensides. The mutant enzymes have been produced by replacing an amino acid residue in the surface part of the parent enzyme with another amino acid residue.  
15 The only mutant enzyme specifically described in EP 525 610 is a protease. Amylase is mentioned as an example of an enzyme which may obtain an improved stability towards ionic tensides, but the type of amylase, its origin or specific mutations are not specified.

WO 94/02597 discloses  $\alpha$ -amylase mutants which exhibit improved stability and  
20 activity in the presence of oxidizing agents. In the mutant  $\alpha$ -amylases, one or more methionine residues have been replaced with amino acid residues different from Cys and Met. The  $\alpha$ -amylase mutants are stated to be useful as detergent and/or dishwashing additives as well as for textile desizing.

WO 94/18314 discloses oxidatively stable  $\alpha$ -amylase mutants, including  
25 mutations in the M197 position of *B. licheniformis*  $\alpha$ -amylase.

EP 368 341 describes the use of pullulanase and other amylolytic enzymes optionally in combination with an  $\alpha$ -amylase for washing and dishwashing.

An object of the present invention is to provide  $\alpha$ -amylase variants which - relative to their parent  $\alpha$ -amylase - possess improved properties of importance, 5 inter alia, in relation to the washing and/or dishwashing performance of the variants in question, e.g. increased thermal stability, increased stability towards oxidation, reduced dependency on  $\text{Ca}^{2+}$  ion and/or improved stability or activity in the pH region of relevance in, e.g., laundry washing or dishwashing. Such variant  $\alpha$ -amylases have the advantage, among others, that they may be 10 employed in a lower dosage than their parent  $\alpha$ -amylase. Furthermore, the  $\alpha$ -amylase variants may be able to remove starchy stains which cannot, or can only with difficulty, be removed by  $\alpha$ -amylase detergent enzymes known today.

#### BRIEF DISCLOSURE OF THE INVENTION

A goal of the work underlying the present invention was to improve, if possible, 15 the stability of, *inter alia*, particular  $\alpha$ -amylases which are obtainable from *Bacillus* strains and which themselves had been selected on the basis of their starch removal performance in alkaline media (such as in detergent solutions as typically employed in laundry washing or dishwashing) relative to many of the currently commercially available  $\alpha$ -amylases. In this connection, the present inventors have 20 surprisingly found that it is in fact possible to improve properties of the types mentioned earlier (*vide supra*) of such a parent  $\alpha$ -amylase by judicial modification of one or more amino acid residues in various regions of the amino acid sequence of the parent  $\alpha$ -amylase. The present invention is based on this finding.

Accordingly, in a first aspect the present invention relates to variants of a parent 25  $\alpha$ -amylase, the parent  $\alpha$ -amylase in question being one which:

i) has one of the amino acid sequences shown in SEQ ID No. 1, SEQ ID No. 2, SEQ ID No. 3 and the sequence numbered 4 in Fig. 2, respectively, herein; or

ii) displays at least 80% homology with one or more of the amino acid sequences shown in SEQ ID No. 1, SEQ ID No. 2, SEQ ID No. 3 and the sequence numbered 4 in Fig. 2, respectively; and/or displays immunological cross-reactivity with an antibody raised against an  $\alpha$ -amylase having one of the amino acid sequences shown in SEQ ID No. 1, SEQ ID No. 2, SEQ ID No. 3 and the sequence numbered 4 in Fig. 2, respectively; and/or is encoded by a DNA sequence which hybridizes with the same probe as a DNA sequence encoding an  $\alpha$ -amylase having one of the amino acid sequences shown in SEQ ID No. 1, SEQ ID No. 2, SEQ ID No. 3 and the sequence numbered 4 in Fig. 2, respectively.

An  $\alpha$ -amylase variant of the invention is subject to the proviso that it is a variant which does not have an amino acid sequence identical to the amino acid sequence shown in SEQ ID No. 1, in SEQ ID No. 2 or in SEQ ID No. 3, or identical to the sequence numbered 4 in Fig. 2.

DNA sequences encoding the first three of the  $\alpha$ -amylase amino acid sequences in question are shown in SEQ ID No. 4 (encoding the amino acid sequence shown in SEQ ID No. 1, SEQ ID No. 5 (encoding the amino acid sequence shown in SEQ ID No. 2) and SEQ ID No. 6 (encoding the amino acid sequence shown in SEQ ID No. 3).

The variants of the invention are variants in which: (a) at least one amino acid residue of the parent  $\alpha$ -amylase has been deleted; and/or (b) at least one amino acid residue of the parent  $\alpha$ -amylase has been replaced (i.e. substituted) by a different amino acid residue; and/or (c) at least one amino acid residue has been inserted relative to the parent  $\alpha$ -amylase. The variants in question have themselves  $\alpha$ -amylase activity and exhibit at least one of the following properties relative to the parent  $\alpha$ -amylase:

increased thermostability, i.e. satisfactory retention of enzymatic activity at a temperature higher than that suitable for use with the parent enzyme;

increased oxidation stability, i.e. increased resistance to degradation by oxidants (such as oxygen, oxidizing bleaching agents and the like);

5 reduced  $\text{Ca}^{2+}$  dependency, i.e. the ability to function satisfactorily in the presence of a lower  $\text{Ca}^{2+}$  concentration than in the case of the parent  $\alpha$ -amylase.  $\alpha$ -Amylases with such reduced  $\text{Ca}^{2+}$  dependency are highly desirable for use in detergent compositions, since such compositions typically contain relatively large amounts of substances (such as phosphates, EDTA and the like) which bind  
10 calcium ions strongly.

Examples of other desirable improvements or modifications of properties (relative to the parent  $\alpha$ -amylase in question) which may be achieved with a variant according to the invention are:

increased stability and/or  $\alpha$ -amylolytic activity at neutral to relatively high pH  
15 values, e.g. at pH values in the range of 7-10.5, such as in the range of 8.5-10.5;

increased  $\alpha$ -amylolytic activity at relatively high temperatures, e.g. temperatures in the range of 40-70°C;

increase or decrease of the isoelectric point (pI) so as to better match the pI value  
20 for the  $\alpha$ -amylase variant in question to the pH of the medium (e.g. a laundry washing medium, dishwashing medium or textile-desizing medium) in which the variant is to be employed (*vide infra*); and

improved binding of a particular type of substrate, improved specificity towards a substrate, and/or improved specificity with respect to cleavage (hydrolysis) of  
25 substrate.

An amino acid sequence is considered to be X% homologous to the parent  $\alpha$ -amylase if a comparison of the respective amino acid sequences, performed via known algorithms, such as the one described by Lipman and Pearson in Science 227 (1985) p. 1435, reveals an identity of X%.

5 In the context of the present invention, "improved performance" as used in connection with washing and dishwashing is, as already indicated above, intended to mean improved removal of starchy stains, i.e. stains containing starch, during washing or dishwashing, respectively. The performance may be determined in conventional washing and dishwashing experiments and the improvement eval-  
10 uated as a comparison with the performance of the parent  $\alpha$ -amylase in question. An example of a small-scale "mini dishwashing test" which can be used as an indicator of dishwashing performance is described in the Experimental section, below.

It will be understood that a variety of different characteristics of the  $\alpha$ -amylase  
15 variant, including specific activity, substrate specificity,  $K_m$ ,  $V_{max}$ ,  $pI$ ,  $pH$  optimum, temperature optimum, thermoactivation, stability towards oxidants and detergents, etc., taken alone or in combination are involved in providing the improved performance. The skilled person will be aware that the performance of the variant cannot, alone, be predicted on the basis of the above characteristics,  
20 but would have to be accompanied by washing and/or dishwashing performance tests.

In further aspects the invention relates to a DNA construct comprising a DNA sequence encoding an  $\alpha$ -amylase variant of the invention, a recombinant expression vector carrying the DNA construct, a cell which is transformed with  
25 the DNA construct or the vector, as well as a method of producing an  $\alpha$ -amylase variant by culturing such a cell under conditions conducive to the production of the  $\alpha$ -amylase variant, after which the  $\alpha$ -amylase variant is recovered from the culture.



In a further aspect the invention relates to a method of preparing a variant of a parent  $\alpha$ -amylase which by virtue of its improved properties as described above exhibits improved washing and/or dishwashing performance as compared to the parent  $\alpha$ -amylase. This method comprises

- 5 a) constructing a population of cells containing genes encoding variants of said parent  $\alpha$ -amylase,
  - b) screening the population of cells for  $\alpha$ -amylase activity under conditions simulating at least one washing and/or dishwashing condition,
  - c) isolating a cell from the population containing a gene encoding a variant of said
  - 10 parent  $\alpha$ -amylase which has improved activity as compared with the parent  $\alpha$ -amylase under the conditions selected in step b),
  - d) culturing the cell isolated in step c) under suitable conditions in an appropriate culture medium, and
  - e) recovering the  $\alpha$ -amylase variant from the culture obtained in step d).
- 15 The invention also relates to a variant (which is a variant according the invention) prepared by the latter method.

In the present context, the term "simulating at least one washing and/or dishwashing condition" is intended to indicate a simulation of, e.g., the temperature or pH prevailing during washing or dishwashing, as well as the

20 chemical composition of a detergent composition to be used in the washing or dishwashing treatment. The term "chemical composition" is intended to include one, or a combination of two or more, constituents of the detergent composition in question. The constituents of a number of different detergent compositions are listed further below.

- 25 The "population of cells" referred to in step a) may suitably be constructed by cloning a DNA sequence encoding a parent  $\alpha$ -amylase and subjecting the DNA to site-directed or random mutagenesis as described herein.

In the present context the term "variant" is used interchangeably with the term "mutant". The term "variant" is intended to include hybrid  $\alpha$ -amylases, i.e.  $\alpha$ -amylases comprising parts of at least two different  $\alpha$ -amylolytic enzymes. Thus, such a hybrid may be constructed, e.g., from: one or more parts each deriving  
5 from a variant as already defined above; or one or more parts each deriving from a variant as already defined above, and one or more parts each deriving from an unmodified parent  $\alpha$ -amylase. In this connection, the invention also relates to a method of producing such a hybrid  $\alpha$ -amylase having improved washing and/or dishwashing performance as compared to any of its constituent enzymes (i.e. as  
10 compared to any of the enzymes which contribute a part to the hybrid), which method comprises:

- a) recombining *in vivo* or *in vitro* the N-terminal coding region of an  $\alpha$ -amylase gene or corresponding cDNA of one of the constituent  $\alpha$ -amylases with the C-terminal coding region of an  $\alpha$ -amylase gene or corresponding cDNA of another  
15 constituent  $\alpha$ -amylase to form recombinants,
- b) selecting recombinants that produce a hybrid  $\alpha$ -amylase having improved washing and/or dishwashing performance as compared to any of its constituent  $\alpha$ -amylases,
- c) culturing recombinants selected in step b) under suitable conditions in an  
20 appropriate culture medium, and
- d) recovering the hybrid  $\alpha$ -amylase from the culture obtained in step c).

In further aspects the invention relates to the use of an  $\alpha$ -amylase variant of the invention [including any variant or hybrid prepared by one of the above mentioned methods] as a detergent enzyme, in particular for washing or dishwashing, to a  
25 detergent additive and a detergent composition comprising the  $\alpha$ -amylase variant, and to the use of an  $\alpha$ -amylase variant of the invention for textile desizing.

Random mutagenesis may be used to generate variants according to the invention, and the invention further relates to a method of preparing a variant of a parent  $\alpha$ -amylase, which method comprises

- (a) subjecting a DNA sequence encoding the parent  $\alpha$ -amylase to random mutagenesis,
- (b) expressing the mutated DNA sequence obtained in step (a) in a host cell, and
- (c) screening for host cells expressing a mutated amylolytic enzyme which has improved properties as described above (e.g. properties such as decreased calcium dependency, increased oxidation stability, increased thermostability, and/or improved activity at relatively high pH) as compared to the parent  $\alpha$ -amylase.

## DETAILED DISCLOSURE OF THE INVENTION

### 10 Nomenclature

In the present description and claims, the conventional one-letter codes for nucleotides and the conventional one-letter and three-letter codes for amino acid residues are used. For ease of reference,  $\alpha$ -amylase variants of the invention are described by use of the following nomenclature:

### 15 Original amino acid(s):position(s):substituted amino acid(s)

According to this nomenclature, and by way of example, the substitution of alanine for asparagine in position 30 is shown as:

Ala 30 Asn                      or                      A30N

### 20 a deletion of alanine in the same position is shown as:

Ala 30 \*                      or                      A30\*

and insertion of an additional amino acid residue, such as lysine, is shown as:

Ala 30 AlaLys                      or                      A30AK

A deletion of a consecutive stretch of amino acid residues, exemplified by amino acid residues 30-33, is indicated as (30-33)\*.

Where a specific  $\alpha$ -amylase contains a "deletion" (i.e. lacks an amino acid residue) in comparison with other  $\alpha$ -amylases and an insertion is made in such a position, this is indicated as:

\* 36 Asp                      or                      \*36D

5 for insertion of an aspartic acid in position 36

Multiple mutations are separated by plus signs, i.e.:

Ala 30 Asp + Glu 34 Ser                      or                      A30N + E34S

representing mutations in positions 30 and 34 (in which alanine and glutamic acid replace, i.e. are substituted for, asparagine and serine, respectively).

10 When one or more alternative amino acid residues may be inserted in a given position this is indicated as:

A30N,E or

A30N or A30E

Furthermore, when a position suitable for modification is identified herein without  
15 any specific modification being suggested, it is to be understood that any other amino acid residue may be substituted for the amino acid residue present in that position (i.e. any amino acid residue - other than that normally present in the position in question - chosen among A, R, N, D, C, Q, E, G, H, I, L, K, M, F, P, S, T, W, Y and V). Thus, for instance, when a modification (replacement) of a  
20 methionine in position 202 is mentioned, but not specified, it is to be understood that any of the other amino acids may be substituted for the methionine, i.e. any other amino acid chosen among A,R,N,D,C,Q,E,G,H,I,L,K,F,P,S,T,W,Y and V.

#### The parent $\alpha$ -amylase

As already indicated, an  $\alpha$ -amylase variant of the invention is very suitably  
25 prepared on the basis of a parent  $\alpha$ -amylase having one of the amino acid sequences shown in SEQ ID No. 1, SEQ ID No. 2, SEQ ID No. 3 and the sequence numbered 4 in Fig. 2, respectively (*vide infra*).

The parent  $\alpha$ -amylases having the amino acid sequences shown in SEQ ID No. 1 and SEQ ID No. 2, respectively, are obtainable from alkaliphilic *Bacillus* strains (strain NCIB 12512 and strain NCIB 12513, respectively), both of which are described in detail in EP 0 277 216 B1 and were deposited in accordance with  
5 the Budapest Treaty on 5 August 1987 at the National Collection of Industrial Bacteria (NCIB) under accession Nos. NCIB 12512 and NCB 12513, respectively. Their preparation from those sources is described in greater detail in the Experimental section herein (*vide infra*).

The parent  $\alpha$ -amylase having the amino acid sequence shown in SEQ ID No. 3 is  
10 obtainable from *Bacillus stearothermophilus* and is described in, inter alia, J. Bacteriol. 166 (1986) pp. 635-643.

The parent  $\alpha$ -amylase having the amino acid sequence numbered 4 in Fig. 2 is obtainable from a "*Bacillus* sp. #707" and is described by Tsukamoto et al. in Biochem. Biophys. Res. Commun. 151 (1988) pp. 25-31.

15 Apart from variants of the above-mentioned parent  $\alpha$ -amylases having the amino acid sequences shown in SEQ ID No. 1, SEQ ID No. 2, SEQ ID No. 3 and the sequence numbered 4 in Fig. 2, respectively, other interesting variants according to the invention include variants of parent  $\alpha$ -amylases which have amino acid sequences exhibiting a high degree of homology, such as at least 70% homology,  
20 preferably (as already indicated) at least 80% homology, desirably at least 85% homology, and more preferably at least 90% homology, e.g.  $\geq 95\%$  homology, with at least one of the latter four amino acid sequences.

As also already indicated above, further criteria for identifying a suitable parent  $\alpha$ -amylase are a) that the  $\alpha$ -amylase displays an immunological cross-reaction  
25 with an antibody raised against an  $\alpha$ -amylase having one of the amino acid sequences shown in SEQ ID No. 1, SEQ ID No. 2, SEQ ID No. 3 and the sequence numbered 4 in Fig. 2, respectively, and/or b) that the  $\alpha$ -amylase is encoded by a DNA sequence which hybridizes with the same probe as a DNA

sequence encoding an  $\alpha$ -amylase having one of the amino acid sequences shown in SEQ ID No. 1, SEQ ID No. 2, SEQ ID No. 3 and the sequence numbered 4 in Fig. 2, respectively.

As already mentioned, with regard to determination of the degree of homology of polypeptides (such as enzymes), amino acid sequence comparisons can be performed using known algorithms, such as the one described by Lipman and Pearson (1985).

Assays for immunological cross-reactivity may be carried out using an antibody raised against, or reactive with, at least one epitope of the  $\alpha$ -amylase having the amino acid sequence shown in SEQ ID No. 1, or of the  $\alpha$ -amylase having the amino acid sequence shown in SEQ ID No. 2, or of the  $\alpha$ -amylase having the amino acid sequence shown in SEQ ID No. 3, or of the  $\alpha$ -amylase having the amino acid sequence numbered 4 in Fig. 2.

The antibody, which may either be monoclonal or polyclonal, may be produced by methods known in the art, e.g. as described by Hudson et al. (1989). Examples of suitable assay techniques well known in the art include Western Blotting and Radial Immunodiffusion Assay, e.g. as described by Hudson et al. (1989).

The oligonucleotide probe for use in the identification of suitable parent  $\alpha$ -amylases on the basis of probe hybridization [criterion b) above] may, by way of example, suitably be prepared on the basis of the full or partial amino acid sequence of an  $\alpha$ -amylase having one of the sequences shown in SEQ ID No. 1, SEQ ID No. 2, SEQ ID No. 3, and the sequence numbered 4 in Fig. 2, respectively, or on the basis of the full or partial nucleotide sequence corresponding thereto.

Suitable conditions for testing hybridization involve presoaking in 5xSSC and pre-hybridizing for 1h at  $\sim 40^{\circ}\text{C}$  in a solution of 20% formamide, 5xDenhardt's

solution, 50mM sodium phosphate, pH 6.8, and 50 $\mu$ g of denatured sonicated calf thymus DNA, followed by hybridization in the same solution supplemented with 100 $\mu$ M ATP for 18h at  $\sim$ 40°C, or using other methods described by, e.g., Sambrook et al. (1989).

#### 5 Influence of mutations on particular properties

From the results obtained by the present inventors it appears that changes in a particular property, e.g. thermal stability or oxidation stability, exhibited by a variant relative to the parent  $\alpha$ -amylase in question can to a considerable extent be correlated with the type of, and positioning of, mutation(s) (amino acid  
10 substitutions, deletions or insertions) in the variant. It is to be understood, however, that the observation that a particular mutation or pattern of mutations leads to changes in a given property in no way excludes the possibility that the mutation(s) in question can also influence other properties.

Oxidation stability: With respect to increasing the oxidation stability of an  
15  $\alpha$ -amylase variant relative to its parent  $\alpha$ -amylase, it appears to be particularly desirable that at least one, and preferably multiple, oxidizable amino acid residue(s) of the parent has/have been deleted or replaced (i.e. substituted by) a different amino acid residue which is less susceptible to oxidation than the original oxidizable amino acid residue.

20 Particularly relevant oxidizable amino acid residues in this connection are cysteine, methionine, tryptophan and tyrosine. Thus, for example, in the case of parent  $\alpha$ -amylases containing cysteine it is anticipated that deletion of cysteine residues, or substitution thereof by less oxidizable amino acid residues, will be of importance in obtaining variants with improved oxidation stability relative to the  
25 parent  $\alpha$ -amylase.

In the case of the above-mentioned parent  $\alpha$ -amylases having the amino acid sequences shown in SEQ ID No. 1, SEQ ID No. 2 and the sequence numbered 4 in Fig. 2, respectively, all of which contain no cysteine residues but have a

significant methionine content, the deletion or substitution of methionine residues is particularly relevant with respect to achieving improved oxidation stability of the resulting variants. Thus, deletion or substitution [e.g. by threonine (T), or by one of the other amino acids listed above] of one or more of the methionine residues in positions M9, M10, M105, M202, M208, M261, M309, M382, M430 and M440 of the amino acid sequences shown in SEQ ID No. 1, SEQ ID No. 2 and the sequence numbered 4 in Fig. 2, and/or in position M323 of the amino acid sequence shown in SEQ ID No. 2 (or deletion or substitution of methionine residues in equivalent positions in the sequence of another  $\alpha$ -amylase meeting one of the other criteria for a parent  $\alpha$ -amylase mentioned above) appear to be particularly effective with respect to increasing the oxidation stability.

In the case of the parent  $\alpha$ -amylase having the amino acid sequence shown in SEQ ID No. 3, relevant amino acid residues which may be deleted or substituted with a view to improving the oxidation stability include the single cysteine residue (C363) and - by analogy with the sequences shown in SEQ ID No. 1 and SEQ ID No. 3 - the methionine residues located in positions M8, M9, M96, M200, M206, M284, M307, M311, M316 and M438.

In this connection, the term "equivalent position" denotes a position which, on the basis of an alignment of the amino acid sequence of the parent  $\alpha$ -amylase in question with the "reference"  $\alpha$ -amylase amino acid sequence in question (for example the sequence shown in SEQ ID No. 1) so as to achieve juxtapositioning of amino acid residues/regions which are common to both, corresponds most closely to (e.g. is occupied by the same amino acid residue as) a particular position in the reference sequence in question.

Particularly interesting mutations in connection with modification of the oxidation stability of the  $\alpha$ -amylases having the amino acid sequences shown in SEQ ID No. 1, SEQ ID No. 2 and the sequence numbered 4 in Fig. 2, respectively, are one or more of the following methionine substitutions (or equivalents thereof in the amino acid sequences of other  $\alpha$ -amylases meeting the requirements of a parent



$\alpha$ -amylase in the context of the invention):  
M202A,R,N,D,Q,E,G,H,I,L,K,F,P,S,T,W,Y,V.

Further relevant methionine substitutions in the amino acid sequence shown in SEQ ID No. 2 are: M323A,R,N,D,Q,E,G,H,I,L,K,F,P,S,T,W,Y,V.

5 Particularly interesting mutations in connection with modification of the oxidation stability of the  $\alpha$ -amylase having the amino acid sequence shown in SEQ ID No. 3 are one or more of the following methionine substitutions:

M 2 0 0 A , R , N , D , Q , E , G , H , I , L , K , F , P , S , T , W , Y , V ;

M 3 1 1 A , R , N , D , Q , E , G , H , I , L , K , F , P , S , T , W , Y , V ; and

10 M316A,R,N,D,Q,E,G,H,I,L,K,F,P,S,T,W,Y,V.

Thermal stability: With respect to increasing the thermal stability of an  $\alpha$ -amylase variant relative to its parent  $\alpha$ -amylase, it appears to be particularly desirable to delete at least one, and preferably two or even three, of the following amino acid residues in the amino acid sequence shown in SEQ ID No. 1 (or their equivalents):  
15 F180, R181, G182, T183, G184 and K185. The corresponding, particularly relevant (and equivalent) amino acid residues in the amino acid sequences shown in SEQ ID No. 2, SEQ ID No. 3 and the sequence numbered 4 in Fig. 2, respectively, are: F180, R181, G182, D183, G184 and K185 (SEQ ID No. 2); F178, R179, G180, I181, G182 and K183 (SEQ ID No. 3); and F180, R181,  
20 G182, H183, G184 and K185 (sequence numbered 4 in Fig. 2).

Particularly interesting pairwise deletions of this type are as follows:

R181\* + G182\*; and T183\* + G184\* (SEQ ID No. 1);

R181\* + G182\*; and D183\* + G184\* (SEQ ID No. 2);

R179\* + G180\*; and I181\* + G182\* (SEQ ID No. 3); and

25 R181\* + G182\*; and H183\* + G184\* (sequence numbered 4 in Fig. 2).

(or equivalents of these pairwise deletions in another  $\alpha$ -amylase meeting the requirements of a parent  $\alpha$ -amylase in the context of the present invention).

Other mutations which appear to be of importance in connection with thermal stability are substitutions of one or more of the amino acid residues from P260 to I275 in the sequence shown in SEQ ID No. 1 (or equivalents thereof in another parent  $\alpha$ -amylase in the context of the invention), such as substitution of the  
5 lysine residue in position 269.

Examples of specific mutations which appear to be of importance in connection with the thermal stability of an  $\alpha$ -amylase variant relative to the parent  $\alpha$ -amylase in question are one or more of the following substitutions in the amino acid sequence shown in SEQ ID No. 1 (or equivalents thereof in another parent  
10  $\alpha$ -amylase in the context of the invention): K269R; P260E; R124P; M105F,I,L,V; M208F,W,Y; L217I; V206I,L,F.

For the parent  $\alpha$ -amylase having the amino acid sequence shown in SEQ ID No. 2, important further (equivalent) mutations are, correspondingly, one or more of the substitutions: M105F,I,L,V; M208F,W,Y; L217I; V206I,L,F; and K269R.

15 For the parent  $\alpha$ -amylase having the amino acid sequence shown in SEQ ID No. 3, important further (equivalent) mutations are, correspondingly, one or both of the substitutions: M206F,W,Y; and L215I.

For the parent  $\alpha$ -amylase having the amino acid sequence numbered 4 in Fig. 2, important further (equivalent) mutations are, correspondingly, one or more of the  
20 substitutions: M105F,I,L,V; M208F,W,Y; L217I; and K269R.

Still further examples of mutations which appear to be of importance, *inter alia*, in achieving improved thermal stability of an  $\alpha$ -amylase variant relative to the parent  $\alpha$ -amylase in question are one or more of the following substitutions in the amino acid sequences shown in SEQ ID No. 1, SEQ ID No. 2 and the sequence  
25 numbered 4 in Fig. 2 (or equivalents thereof in another parent  $\alpha$ -amylase in the context of the invention): A354C + V479C; L351C + M430C; N457D,E + K385R; L355D,E + M430R,K; L355D,E + I411R,K; and N457D,E.

Ca<sup>2+</sup> dependency: With respect to achieving decreased Ca<sup>2+</sup> dependency of an  $\alpha$ -amylase variant relative to its parent  $\alpha$ -amylase [i.e. with respect to obtaining a variant which exhibits satisfactory amylolytic activity in the presence of a lower concentration of calcium ion in the extraneous medium than is necessary for the parent enzyme, and which, for example, therefore is less sensitive than the parent to calcium ion-depleting conditions such as those obtaining in media containing calcium-complexing agents (such as certain detergent builders)], it appears to be particularly desirable to incorporate one or more of the following substitutions in the amino acid sequences shown in SEQ ID No. 1, SEQ ID No. 2 and the sequence numbered 4 in Fig. 2 (or an equivalent substitution in another parent  $\alpha$ -amylase in the context of the invention): Y243F, K108R, K179R, K239R, K242R, K269R, D163N, D188N, D192N, D199N, D205N, D207N, D209N, E190Q, E194Q and N106D.

In the case of the amino acid sequence shown in SEQ ID No. 3, particularly desirable substitutions appear, correspondingly, to be one or more of the following: K107R, K177R, K237R, K240R, D162N, D186N, D190N, D197N, D203N, D205N, D207N, E188Q and E192Q.

As well as the above-mentioned replacements of D residues with N residues, or of E residues with Q residues, other relevant substitutions in the context of reducing Ca<sup>2+</sup> dependency are replacement of the D and/or E residues in question with any other amino acid residue.

Further substitutions which appear to be of importance in the context of Ca<sup>2+</sup> dependency are pairwise substitutions of the amino acid residues present at positions 113 and 151 in the amino acid sequences shown in SEQ ID No. 1, SEQ ID No. 2, and in the sequence numbered 4 in Fig. 2, and at positions 112 and 150 in the amino acid sequence shown in SEQ ID No. 3 (or equivalent pairwise substitutions in another parent  $\alpha$ -amylase in the context of the invention), i.e. pairwise substitutions of the following amino acid residues:

G113 + N151 (in relation to SEQ ID No.1); A113 + T151 (in relation to SEQ ID No. 2 and to the sequence numbered 4 in Fig. 2); and G112 + T150 (in relation to SEQ ID No. 3).

Particularly interesting pairwise substitutions of this type with respect to achieving decreased  $\text{Ca}^{2+}$  dependency are the following:

G113T + N151I (in relation to SEQ ID No.1); A113T + T151I (in relation to SEQ ID No. 2 and to the sequence numbered 4 in Fig. 2); and G112T + T150I (in relation to SEQ ID No. 3).

In connection with substitutions of relevance for  $\text{Ca}^{2+}$  dependency, some other substitutions which appear to be of importance in stabilizing the enzyme conformation, and which it is contemplated may achieve this by, e.g., enhancing the strength of binding or retention of calcium ion at or within a calcium binding site within the  $\alpha$ -amylolytic enzyme, are one or more of the following substitutions in the amino acid sequences shown in SEQ ID No. 1, SEQ ID No. 2 and the sequence numbered 4 in Fig. 2 (or an equivalent substitution in another parent  $\alpha$ -amylase in the context of the invention): G304W,F,Y,R,I,L,V,Q,N; G305A,S,N,D,Q,E,R,K; and H408Q,E.

Corresponding (equivalent) substitutions in the amino acid sequence shown in SEQ ID No. 3 are: G302W,F,Y,R,I,L,V,Q,N; and G303A,S,N,D,Q,E,R,K.

Isoelectric point (pI): Preliminary results indicate that the washing performance, e.g. the laundry washing performance, of an  $\alpha$ -amylase is optimal when the pH of the washing liquor (washing medium) is close to the pI value for the  $\alpha$ -amylase in question. It will thus be desirable, where appropriate, to produce an  $\alpha$ -amylase variant having an isoelectric point (pI value) which is better matched to the pH of a medium (such as a washing medium) in which the enzyme is to be employed than the isoelectric point of the parent  $\alpha$ -amylase in question.

With respect to decreasing the isoelectric point, preferred mutations in the amino acid sequence shown in SEQ ID No. 1 include one or more of the following substitutions: Q86E, R124P, S154D, T183D, V222E, P260E, R310A, Q346E, Q391E, N437E, K444Q and R452H. Appropriate combinations of these  
5 substitutions in the context of decreasing the isoelectric point include: Q391E + K444Q; and Q391E + K444Q + S154D.

Correspondingly, preferred mutations in the amino acid sequence shown in SEQ ID No. 3 with respect to decreasing the isoelectric point include one or more of the substitutions: L85E, S153D, I181D, K220E, P258E, R308A, P344E, Q358E  
10 and S435E.

With respect to increasing the isoelectric point, preferred mutations in the amino acid sequence shown in SEQ ID No. 2 include one or more of the following substitutions: E86Q,L; D154S; D183T,I; E222V,K; E260P; A310R; E346Q,P; E437N,S; and H452R.

15 In the Experimental section below, the construction of a number of variants according to the invention is described.

$\alpha$ -Amylase variants of the invention will, apart from having one or more improved properties as discussed above, preferably be such that they have a higher starch hydrolysis velocity at low substrate concentrations than the parent  $\alpha$ -amylase.  
20 Alternatively, an  $\alpha$ -amylase variant of the invention will preferably be one which has a higher  $V_{max}$  and/or a lower  $K_m$  than the parent  $\alpha$ -amylase when tested under the same conditions. In the case of a hybrid  $\alpha$ -amylase, the "parent  $\alpha$ -amylase" to be used for the comparison should be the one of the constituent enzymes having the best performance.

25 The  $V_{max}$ ,  $K_m$  and  $V$  may be determined by well-known procedures.

### Methods of preparing $\alpha$ -amylase variants

Several methods for introducing mutations into genes are known in the art. After a brief discussion of the cloning of  $\alpha$ -amylase-encoding DNA sequences, methods for generating mutations at specific sites within the  $\alpha$ -amylase-encoding sequence  
5 will be discussed.

### Cloning a DNA sequence encoding an $\alpha$ -amylase

The DNA sequence encoding a parent  $\alpha$ -amylase may be isolated from any cell or microorganism producing the  $\alpha$ -amylase in question, using various methods well known in the art. First, a genomic DNA and/or cDNA library should be  
10 constructed using chromosomal DNA or messenger RNA from the organism that produces the  $\alpha$ -amylase to be studied. Then, if the amino acid sequence of the  $\alpha$ -amylase is known, homologous, labelled oligonucleotide probes may be synthesized and used to identify  $\alpha$ -amylase-encoding clones from a genomic library prepared from the organism in question. Alternatively, a labelled oligonu-  
15 cleotide probe containing sequences homologous to a known  $\alpha$ -amylase gene could be used as a probe to identify  $\alpha$ -amylase-encoding clones, using hybridization and washing conditions of lower stringency.

Yet another method for identifying  $\alpha$ -amylase-encoding clones would involve inserting fragments of genomic DNA into an expression vector, such as a  
20 plasmid, transforming  $\alpha$ -amylase-negative bacteria with the resulting genomic DNA library, and then plating the transformed bacteria onto agar containing a substrate for  $\alpha$ -amylase, thereby allowing clones expressing the  $\alpha$ -amylase to be identified.

Alternatively, the DNA sequence encoding the enzyme may be prepared  
25 synthetically by established standard methods, e.g. the phosphoamidite method described by S.L. Beaucage and M.H. Caruthers (1981) or the method described by Matthes et al. (1984). In the phosphoamidite method, oligonucleotides are synthesized, e.g. in an automatic DNA synthesizer, purified, annealed, ligated and cloned in appropriate vectors.

Finally, the DNA sequence may be of mixed genomic and synthetic origin, mixed synthetic and cDNA origin or mixed genomic and cDNA origin, prepared by ligating fragments of synthetic, genomic or cDNA origin (as appropriate, the fragments corresponding to various parts of the entire DNA sequence), in accordance with standard techniques. The DNA sequence may also be prepared by polymerase chain reaction (PCR) using specific primers, for instance as described in US 4,683,202 or R.K. Saiki et al. (1988).

#### Site-directed mutagenesis

Once an  $\alpha$ -amylase-encoding DNA sequence has been isolated, and desirable sites for mutation identified, mutations may be introduced using synthetic oligonucleotides. These oligonucleotides contain nucleotide sequences flanking the desired mutation sites; mutant nucleotides are inserted during oligonucleotide synthesis. In a specific method, a single-stranded gap of DNA, bridging the  $\alpha$ -amylase-encoding sequence, is created in a vector carrying the  $\alpha$ -amylase gene. Then the synthetic nucleotide, bearing the desired mutation, is annealed to a homologous portion of the single-stranded DNA. The remaining gap is then filled in with DNA polymerase I (Klenow fragment) and the construct is ligated using T4 ligase. A specific example of this method is described in Morinaga et al. (1984). US 4,760,025 discloses the introduction of oligonucleotides encoding multiple mutations by performing minor alterations of the cassette. However, an even greater variety of mutations can be introduced at any one time by the Morinaga method, because a multitude of oligonucleotides, of various lengths, can be introduced.

Another method of introducing mutations into  $\alpha$ -amylase-encoding DNA sequences is described in Nelson and Long (1989). It involves the 3-step generation of a PCR fragment containing the desired mutation introduced by using a chemically synthesized DNA strand as one of the primers in the PCR reactions. From the PCR-generated fragment, a DNA fragment carrying the mutation may be isolated by cleavage with restriction endonucleases and reinserted into an expression plasmid.

Random mutagenesis

Random mutagenesis is suitably performed either as localized or region-specific random mutagenesis in at least three parts of the gene translating to the amino acid sequence shown in question, or within the whole gene.

- 5 For region-specific random mutagenesis with a view to improving the thermal stability, the following codon positions, in particular, may appropriately be targetted (using one-letter amino acid abbreviations and the numbering of the amino acid residues in the sequence in question):

In the amino acid sequence shown in SEQ ID No. 1:

- 10 120-140 = VEVNRSNRNQETSGEYAIEAW  
 178-187 = YKFRGTGKAW  
 264-277 = VAEFWKNDLGALEN

In the amino acid sequence shown in SEQ ID No. 2:

- 120-140 = VEVNPNRRNQEISGDYTIEAW  
 15 178-187 = YKFRGDGKAW  
 264-277 = VAEFWKNDLGALEN

In the amino acid sequence shown in SEQ ID No. 3:

- 119-139 = VEVNPSDRNQEISGTYQIQAW  
 176-185 = YKFRGIGKAW  
 20 262-275 = VGEYWSYDINKLHN

In the amino acid sequence numbered 4 in Fig. 2:

- 120-140 = VEVNPNRRNQEVTGEYTIEAW  
 178-187 = YKFRGHGKAW  
 264-277 = VAEFWKNDLGALEN

- 25 With a view to achieving reduced  $\text{Ca}^{2+}$  dependency, the following codon positions, in particular, may appropriately be targetted:



In the amino acid sequence shown in SEQ ID No. 1:

178-209 = YKFRGTGKAWDWEVDTENGNYDYLMYADVDM

237-246 = AVKHIKYSFT

In the amino acid sequence shown in SEQ ID No. 2:

5 178-209 = YKFRGDGKAWDWEVDSENGNYDYLMYADVDM

237-246 = AVKHIKYSFT

In the amino acid sequence numbered 4 in Fig. 2:

178-209 = YKFRGHGKAWDWEVDTENGNYDYLMYADIDMD

237-246 = AVKHIKYSFT

- 10 With a view to achieving improved binding of a substrate (i.e. improved binding of a carbohydrate species - such as amylose or amylopectin - which is a substrate for  $\alpha$ -amylolytic enzymes) by an  $\alpha$ -amylase variant, modified (e.g. higher) substrate specificity and/or modified (e.g. higher) specificity with respect to cleavage (hydrolysis) of substrate, it appears that the following codon positions
- 15 for the amino acid sequence shown in SEQ ID No. 1 (or equivalent codon positions for another parent  $\alpha$ -amylase in the context of the invention) may particularly appropriately be targetted:

In the amino acid sequence shown in SEQ ID No. 1:

15-20 = WYLPND

20 52-58 = SQNDVGY

72-78 = KGTVRTK

104-111 = VMNHKGGA

165-174 = TDWDQSRQLQ

194-204 = ENGNYDYLMYA

25 234-240 = RIDAVKH

332-340 = HDSQPGEAL

The random mutagenesis of a DNA sequence encoding a parent  $\alpha$ -amylase to be performed in accordance with step a) of the above-described method of the invention may conveniently be performed by use of any method known in the art.

For instance, the random mutagenesis may be performed by use of a suitable  
5 physical or chemical mutagenizing agent, by use of a suitable oligonucleotide, or by subjecting the DNA sequence to PCR generated mutagenesis. Furthermore, the random mutagenesis may be performed by use of any combination of these mutagenizing agents.

The mutagenizing agent may, e.g., be one which induces transitions,  
10 transversions, inversions, scrambling, deletions, and/or insertions.

Examples of a physical or chemical mutagenizing agent suitable for the present purpose include ultraviolet (UV) irradiation, hydroxylamine, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), O-methyl hydroxylamine, nitrous acid, ethyl methane sulphonate (EMS), sodium bisulphite, formic acid, and nucleotide analogues.

15 When such agents are used, the mutagenesis is typically performed by incubating the DNA sequence encoding the parent enzyme to be mutagenized in the presence of the mutagenizing agent of choice under suitable conditions for the mutagenesis to take place, and selecting for mutated DNA having the desired properties.

20 When the mutagenesis is performed by the use of an oligonucleotide, the oligonucleotide may be doped or spiked with the three non-parent nucleotides during the synthesis of the oligonucleotide at the positions which are to be changed. The doping or spiking may be done so that codons for unwanted amino acids are avoided. The doped or spiked oligonucleotide can be incorporated into  
25 the DNA encoding the amylolytic enzyme by any published technique, using e.g. PCR, LCR or any DNA polymerase and ligase.

When PCR-generated mutagenesis is used, either a chemically treated or non-treated gene encoding a parent  $\alpha$ -amylase enzyme is subjected to PCR under conditions that increase the misincorporation of nucleotides (Deshler 1992; Leung et al., Technique, Vol.1, 1989, pp. 11-15).

- 5 A mutator strain of *E. coli* (Fowler et al., Molec. Gen. Genet., 133, 1974, pp. 179-191), *S. cerevisiae* or any other microbial organism may be used for the random mutagenesis of the DNA encoding the amylolytic enzyme by e.g. transforming a plasmid containing the parent enzyme into the mutator strain, growing the mutator strain with the plasmid and isolating the mutated plasmid  
10 from the mutator strain. The mutated plasmid may subsequently be transformed into the expression organism.

The DNA sequence to be mutagenized may conveniently be present in a genomic or cDNA library prepared from an organism expressing the parent amylolytic enzyme. Alternatively, the DNA sequence may be present on a suitable vector  
15 such as a plasmid or a bacteriophage, which as such may be incubated with or otherwise exposed to the mutagenizing agent. The DNA to be mutagenized may also be present in a host cell either by being integrated in the genome of said cell or by being present on a vector harboured in the cell. Finally, the DNA to be mutagenized may be in isolated form. It will be understood that the DNA  
20 sequence to be subjected to random mutagenesis is preferably a cDNA or a genomic DNA sequence.

In some cases it may be convenient to amplify the mutated DNA sequence prior to the expression step (b) or the screening step (c) being performed. Such amplification may be performed in accordance with methods known in the art,  
25 the presently preferred method being PCR-generated amplification using oligonucleotide primers prepared on the basis of the DNA or amino acid sequence of the parent enzyme.

Subsequent to the incubation with or exposure to the mutagenizing agent, the mutated DNA is expressed by culturing a suitable host cell carrying the DNA sequence under conditions allowing expression to take place. The host cell used for this purpose may be one which has been transformed with the mutated DNA sequence, optionally present on a vector, or one which was carried the DNA sequence encoding the parent enzyme during the mutagenesis treatment. Examples of suitable host cells are the following: grampositive bacteria such as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus lentus*, *Bacillus brevis*, *Bacillus stearothermophilus*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus lautus*, *Bacillus megaterium*, *Bacillus thuringiensis*, *Streptomyces lividans* or *Streptomyces murinus*; and gramnegative bacteria such as *E.coli*.

The mutated DNA sequence may further comprise a DNA sequence encoding functions permitting expression of the mutated DNA sequence.

15 Localized random mutagenesis: the random mutagenesis may advantageously be localized to a part of the parent  $\alpha$ -amylase in question. This may, e.g., be advantageous when certain regions of the enzyme have been identified to be of particular importance for a given property of the enzyme, and when modified are expected to result in a variant having improved properties. Such regions may normally be identified when the tertiary structure of the parent enzyme has been elucidated and related to the function of the enzyme.

The localized random mutagenesis is conveniently performed by use of PCR-generated mutagenesis techniques as described above or any other suitable technique known in the art.

25 Alternatively, the DNA sequence encoding the part of the DNA sequence to be modified may be isolated, e.g. by being inserted into a suitable vector, and said part may subsequently be subjected to mutagenesis by use of any of the mutagenesis methods discussed above.

With respect to the screening step in the above-mentioned method of the invention, this may conveniently be performed by use of a filter assay based on the following principle:

A microorganism capable of expressing the mutated amylolytic enzyme of interest is incubated on a suitable medium and under suitable conditions for the enzyme to be secreted, the medium being provided with a double filter comprising a first protein-binding filter and on top of that a second filter exhibiting a low protein binding capability. The microorganism is located on the second filter. Subsequent to the incubation, the first filter comprising enzymes secreted from the microorganisms is separated from the second filter comprising the microorganisms. The first filter is subjected to screening for the desired enzymatic activity and the corresponding microbial colonies present on the second filter are identified.

The filter used for binding the enzymatic activity may be any protein binding filter e.g. nylon or nitrocellulose. The topfilter carrying the colonies of the expression organism may be any filter that has no or low affinity for binding proteins e.g. cellulose acetate or Durapore™. The filter may be pretreated with any of the conditions to be used for screening or may be treated during the detection of enzymatic activity.

The enzymatic activity may be detected by a dye, fluorescence, precipitation, pH indicator, IR-absorbance or any other known technique for detection of enzymatic activity.

The detecting compound may be immobilized by any immobilizing agent e.g. agarose, agar, gelatine, polyacrylamide, starch, filter paper, cloth; or any combination of immobilizing agents.

$\alpha$ -Amylase activity is detected by Cibacron Red labelled amylopectin, which is immobilized on agarose. For screening for variants with increased thermal and

high-pH stability, the filter with bound  $\alpha$ -amylase variants is incubated in a buffer at pH 10.5 and 60° or 65°C for a specified time, rinsed briefly in deionized water and placed on the amylopectin-agarose matrix for activity detection. Residual activity is seen as lysis of Cibacron Red by amylopectin degradation. The  
5 conditions are chosen to be such that activity due to the  $\alpha$ -amylase having the amino acid sequence shown in SEQ ID No.1 can barely be detected. Stabilized variants show, under the same conditions, increased colour intensity due to increased liberation of Cibacron Red.

For screening for variants with an activity optimum at a lower temperature and/or  
10 over a broader temperature range, the filter with bound variants is placed directly on the amylopectin-Cibacron Red substrate plate and incubated at the desired temperature (e.g. 4°C, 10°C or 30°C) for a specified time. After this time activity due to the  $\alpha$ -amylase having the amino acid sequence shown in SEQ ID No.1 can barely be detected, whereas variants with optimum activity at a lower  
15 temperature will show increase amylopectin lysis. Prior to incubation onto the amylopectin matrix, incubation in all kinds of desired media - e.g. solutions containing  $\text{Ca}^{2+}$ , detergents, EDTA or other relevant additives - can be carried out in order to screen for changed dependency or for reaction of the variants in question with such additives.

## 20 Methods of preparing hybrid $\alpha$ -amylases

As an alternative to site-specific mutagenesis,  $\alpha$ -amylase variants which are hybrids of at least two constituent  $\alpha$ -amylases may be prepared by combining the relevant parts of the respective genes in question.

Naturally occurring enzymes may be genetically modified by random or site  
25 directed mutagenesis as described above. Alternatively, part of one enzyme may be replaced by a part of another to obtain a chimeric enzyme. This replacement can be achieved either by conventional *in vitro* gene splicing techniques or by *in vivo* recombination or by combinations of both techniques. When using conventional *in vitro* gene splicing techniques, a desired portion of the  $\alpha$ -amylase

gene coding sequence may be deleted using appropriate site-specific restriction enzymes; the deleted portion of the coding sequence may then be replaced by the insertion of a desired portion of a different  $\alpha$ -amylase coding sequence so that a chimeric nucleotide sequence encoding a new  $\alpha$ -amylase is produced.

5 Alternatively,  $\alpha$ -amylase genes may be fused, e.g. by use of the PCR overlay extension method described by Higuchi et al. 1988.

The *in vivo* recombination techniques depend on the fact that different DNA segments with highly homologous regions (identity of DNA sequence) may recombine, i.e. break and exchange DNA, and establish new bonds in the

10 homologous regions. Accordingly, when the coding sequences for two different but homologous amylase enzymes are used to transform a host cell, recombination of homologous sequences *in vivo* will result in the production of chimeric gene sequences. Translation of these coding sequences by the host cell will result in production of a chimeric amylase gene product. Specific *in vivo*

15 recombination techniques are described in US 5,093,257 and EP 252 666.

Alternatively, the hybrid enzyme may be synthesized by standard chemical methods known in the art. For example, see Hunkapiller et al. (1984). Accordingly, peptides having the appropriate amino acid sequences may be synthesized in whole or in part and joined to form hybrid enzymes (variants) of the

20 invention.

#### Expression of $\alpha$ -amylase variants

According to the invention, a mutated  $\alpha$ -amylase-encoding DNA sequence produced by methods described above, or by any alternative methods known in the art, can be expressed, in enzyme form, using an expression vector which

25 typically includes control sequences encoding a promoter, operator, ribosome binding site, translation initiation signal, and, optionally, a repressor gene or various activator genes.

The recombinant expression vector carrying the DNA sequence encoding an  $\alpha$ -amylase variant of the invention may be any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may  
5 be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid, a bacteriophage or an extrachromosomal element, minichromosome or an artificial chromosome. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell  
10 genome and replicated together with the chromosome(s) into which it has been integrated.

In the vector, the DNA sequence should be operably connected to a suitable promoter sequence. The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes  
15 encoding proteins either homologous or heterologous to the host cell. Examples of suitable promoters for directing the transcription of the DNA sequence encoding an  $\alpha$ -amylase variant of the invention, especially in a bacterial host, are the promoter of the *lac* operon of *E.coli*, the *Streptomyces coelicolor* agarase gene *dagA* promoters, the promoters of the *Bacillus licheniformis*  $\alpha$ -amylase gene  
20 (*amyL*), the promoters of the *Bacillus stearothermophilus* maltogenic amylase gene (*amyM*), the promoters of the *Bacillus Amyloliquefaciens*  $\alpha$ -amylase (*amyQ*), the promoters of the *Bacillus subtilis* xylA and xylB genes etc. For transcription in a fungal host, examples of useful promoters are those derived from the gene encoding *A. oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *A.*  
25 *niger* neutral  $\alpha$ -amylase, *A. niger* acid stable  $\alpha$ -amylase, *A. niger* glucoamylase, *Rhizomucor miehei* lipase, *A. oryzae* alkaline protease, *A. oryzae* triose phosphate isomerase or *A. nidulans* acetamidase.

The expression vector of the invention may also comprise a suitable transcription terminator and, in eukaryotes, polyadenylation sequences operably connected to  
30 the DNA sequence encoding the  $\alpha$ -amylase variant of the invention. Termination



and polyadenylation sequences may suitably be derived from the same sources as the promoter.

The vector may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. Examples of such sequences are the origins of replication of plasmids pUC19, pACYC177, pUB110, pE194, pAMB1 and pIJ702.

The vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect in the host cell, such as the *dal* genes from *B. subtilis* or *B. licheniformis*, or one which confers antibiotic resistance such as ampicillin, kanamycin, chloramphenicol or tetracycline resistance. Furthermore, the vector may comprise *Aspergillus* selection markers such as amdS, argB, niaD and sC, a marker giving rise to hygromycin resistance, or the selection may be accomplished by co-transformation, e.g. as described in WO 91/17243.

While intracellular expression may be advantageous in some respects, e.g. when using certain bacteria as host cells, it is generally preferred that the expression is extracellular.

Procedures suitable for constructing vectors of the invention encoding an  $\alpha$ -amylase variant, and containing the promoter, terminator and other elements, respectively, are well known to persons skilled in the art [cf., for instance, Sambrook et al. (1989)].

The cell of the invention, either comprising a DNA construct or an expression vector of the invention as defined above, is advantageously used as a host cell in the recombinant production of an  $\alpha$ -amylase variant of the invention. The cell may be transformed with the DNA construct of the invention encoding the variant, conveniently by integrating the DNA construct (in one or more copies) in the host chromosome. This integration is generally considered to be an advantage as the DNA sequence is more likely to be stably maintained in the cell. Integration of the DNA constructs into the host chromosome may be performed

according to conventional methods, e.g. by homologous or heterologous recombination. Alternatively, the cell may be transformed with an expression vector as described above in connection with the different types of host cells.

The cell of the invention may be a cell of a higher organism such as a mammal or an insect, but is preferably a microbial cell, e.g. a bacterial or a fungal (including yeast) cell.

Examples of suitable bacteria are grampositive bacteria such as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus lentus*, *Bacillus brevis*, *Bacillus stearothermophilus*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus lautus*, *Bacillus megaterium*, *Bacillus thuringiensis*, or *Streptomyces lividans* or *Streptomyces murinus*, or gramnegative bacteria such as *E.coli*. The transformation of the bacteria may, for instance, be effected by protoplast transformation or by using competent cells in a manner known *per se*.

The yeast organism may favourably be selected from a species of *Saccharomyces* or *Schizosaccharomyces*, e.g. *Saccharomyces cerevisiae*. The filamentous fungus may advantageously belong to a species of *Aspergillus*, e.g. *Aspergillus oryzae* or *Aspergillus niger*. Fungal cells may be transformed by a process involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a manner known *per se*. A suitable procedure for transformation of *Aspergillus* host cells is described in EP 238 023.

In a yet further aspect, the present invention relates to a method of producing an  $\alpha$ -amylase variant of the invention, which method comprises cultivating a host cell as described above under conditions conducive to the production of the variant and recovering the variant from the cells and/or culture medium.

The medium used to cultivate the cells may be any conventional medium suitable for growing the host cell in question and obtaining expression of the  $\alpha$ -amylase variant of the invention. Suitable media are available from commercial suppliers

or may be prepared according to published recipes (e.g. as described in catalogues of the American Type Culture Collection).

The  $\alpha$ -amylase variant secreted from the host cells may conveniently be recovered from the culture medium by well-known procedures, including  
5 separating the cells from the medium by centrifugation or filtration, and precipitating proteinaceous components of the medium by means of a salt such as ammonium sulphate, followed by the use of chromatographic procedures such as ion exchange chromatography, affinity chromatography, or the like.

#### Industrial Applications

10 Owing to their activity at alkaline pH values,  $\alpha$ -amylase variants of the invention are well suited for use in a variety of industrial processes. In particular, they find potential applications as a component in washing, dishwashing and hard surface cleaning detergent compositions (*vide infra*), but may also be useful in the production of sweeteners and ethanol from starch. Conditions for conventional  
15 starch-converting processes and liquefaction and/or saccharification processes are described in, for instance, US 3,912,590, EP 252,730 and EP 63,909.  $\alpha$ -Amylase variants of the invention also possess properties of value in the production of lignocellulosic materials, such as pulp, paper and cardboard, from starch-reinforced waste paper and waste cardboard, especially where repulping  
20 occurs at a pH above 7, and where amylases can facilitate the disintegration of the waste material through degradation of the reinforcing starch.  $\alpha$ -Amylase variants of the invention are well suited for use in the deinking/recycling processes of making paper out of starch-coated or starch-containing waste printed paper. It is usually desirable to remove the printing ink in order to produce  
25 new paper of high brightness; examples of how the variants of the invention may be used in this way are described in PCT/DK94/00437.

The  $\alpha$ -amylase variants of the invention may also be very useful in modifying starch where enzymatically modified starch is used in papermaking together with alkaline fillers such as calcium carbonate, kaolin and clays. With the alkaline

$\alpha$ -amylase variants of the invention it is feasible to modify the starch in the presence of the filler, thus allowing for a simpler, integrated process.

The  $\alpha$ -amylase variants of the invention are also well suited for use in textile desizing. In the textile processing industry,  $\alpha$ -amylases are traditionally used as  
5 auxiliaries in the desizing process to facilitate the removal of starch-containing size which has served as a protective coating on weft yarns during weaving. Complete removal of the size coating after weaving is important to ensure optimum results in subsequent processes in which the fabric is scoured, bleached and dyed. Enzymatic starch degradation is preferred because it does not harm the  
10 fibres of the textile or fabric.

In order to reduce processing costs and increase mill throughput, the desizing processing is sometimes combined with the scouring and bleaching steps. In such cases, non-enzymatic auxiliaries such as alkali or oxidation agents are typically used to break down the starch, because traditional  $\alpha$ -amylases are not very  
15 compatible with high pH levels and bleaching agents. The non-enzymatic breakdown of the starch size does lead to some fibre damage because of the rather aggressive chemicals used.

Accordingly, it would be desirable to use an  $\alpha$ -amylase variant of the invention, having improved performance in alkaline solution. The  $\alpha$ -amylase variant may be  
20 used alone or in combination with a cellulase when desizing cellulose-containing fabric or textile.

$\alpha$ -Amylase variants of the invention are also believed to be very useful in beer-making processes; in such processes the variants will typically be added during the mashing process.

## 25 Detergent Additive and Composition for Dishwashing and Washing

Due to the improved washing and/or dishwashing performance which will often be a consequence of improvements in properties as discussed above, numerous

$\alpha$ -amylase variants (including hybrids) of the invention are particularly well suited for incorporation into detergent compositions, e.g. detergent compositions intended for performance in the range of pH 7-13, particularly the range of pH 8-11. According to the invention, the  $\alpha$ -amylase variant may be added as a component of a detergent composition. As such, it may be included in the detergent composition in the form of a detergent additive. The detergent composition as well as the detergent additive may additionally comprise one or more other enzymes conventionally used in detergents, such as proteases, lipases, amylolytic enzymes, oxidases (including peroxidases), or cellulases.

10 It has been found that substantial improvements in washing and/or dishwashing performance may be obtained when  $\alpha$ -amylase is combined with another amylolytic enzyme, such as a pullulanase, an iso-amylase, a beta-amylase, an amyloglucosidase or a CTGase. Examples of commercially available amylolytic enzymes suitable for the given purpose are AMG®, Novamyl® and Promozyme®,  
15 all available from Novo Nordisk A/S.

Accordingly, in a particular embodiment the invention relates to a detergent additive comprising an  $\alpha$ -amylase variant of the invention in combination with at least one other amylolytic enzyme (e.g. chosen amongst those mentioned above).

In a specific aspect, the invention provides a detergent additive. The enzymes  
20 may be included in a detergent composition by adding separate additives containing one or more enzymes, or by adding a combined additive comprising all of these enzymes. A detergent additive of the invention, i.e. a separated additive or a combined additive, can be formulated, e.g., as a granulate, liquid, slurry, etc. Preferred detergent additive formulations are granulates (in particular  
25 non-dusting granulates), liquids (in particular stabilized liquids), slurries or protected enzymes.

Non-dusting granulates may be produced, e.g., as disclosed in US 4,106,991 and US 4,661,452, and may optionally be coated by methods known in the art. The detergent enzymes may be mixed before or after granulation.

Liquid enzyme preparations may, for instance, be stabilized by adding a polyol such as propylene glycol, a sugar or sugar alcohol, lactic acid or boric acid according to established methods. Other enzyme stabilizers are well known in the art. Protected enzymes may be prepared according to the method disclosed in EP 238 216.

In a still further aspect, the invention relates to a detergent composition comprising an  $\alpha$ -amylase variant (including hybrid) of the invention.

The detergent composition of the invention may be in any convenient form, e.g. as powder, granules or liquid. A liquid detergent may be aqueous, typically containing up to 90% of water and 0-20% of organic solvent, or non-aqueous, e.g. as described in EP Patent 120,659.

#### 15 Detergent Compositions

An  $\alpha$ -amylase variant of the invention may typically be a component of a detergent composition, e.g., a laundry detergent composition or a dishwashing detergent composition. As such, it may be included in the detergent composition in the form of a non-dusting granulate, a stabilized liquid, or a protected enzyme.

20 Non-dusting granulates may be produced, e.g., as disclosed in US 4,106,991 and 4,661,452 (both to Novo Industri A/S) and may optionally be coated by methods known in the art. Examples of waxy coating materials are poly(ethylene oxide) products (polyethyleneglycol, PEG) with mean molecular weights of 1000 to 20000; ethoxylated nonylphenols having from 16 to 50 ethylene oxide units; 25 ethoxylated fatty alcohols in which the alcohol contains from 12 to 20 carbon atoms and in which there are 15 to 80 ethylene oxide units; fatty alcohols; fatty acids; and mono- and di- and triglycerides of fatty acids. Examples of film-forming coating materials suitable for application by fluid bed techniques are given in

patent GB 1483591. Liquid enzyme preparations may, for instance, be stabilized by adding a polyol such as propylene glycol, a sugar or sugar alcohol, lactic acid or boric acid according to established methods. Other enzyme stabilizers are well known in the art. Protected enzymes may be prepared according to the method  
5 disclosed in EP 238,216.

The detergent composition of the invention may be in any convenient form, e.g. as powder, granules, paste or liquid. A liquid detergent may be aqueous, typically containing up to 70% water and 0-30% organic solvent, or nonaqueous.

The detergent composition comprises one or more surfactants, each of which  
10 may be anionic, nonionic, cationic, or amphoteric (zwitterionic). The detergent will usually contain 0-50% of anionic surfactant such as linear alkylbenzene-sulfonate (LAS), alpha-olefinsulfonate (AOS), alkyl sulfate (fatty alcohol sulfate) (AS), alcohol ethoxysulfate (AEOS or AES), secondary alkanesulfonates (SAS), alpha-sulfo fatty acid methyl esters, alkyl- or alkenylsuccinic acid, or soap. It may  
15 also contain 0-40% of nonionic surfactant such as alcohol ethoxylate (AEO or AE), alcohol propoxylate, carboxylated alcohol ethoxylates, nonylphenol ethoxylate, alkylpolyglycoside, alkyldimethylamine oxide, ethoxylated fatty acid monoethanolamide, fatty acid monoethanolamide, or polyhydroxy alkyl fatty acid amide (e.g. as described in WO 92/06154).

20 The detergent composition may additionally comprise one or more other enzymes, such as pullulanase, esterase, lipase, cutinase, protease, cellulase, peroxidase, or oxidase, e.g., laccase.

Normally the detergent contains 1-65% of a detergent builder (although some dishwashing detergents may contain even up to 90% of a detergent builder) or  
25 complexing agent such as zeolite, diphosphate, triphosphate, phosphonate, citrate, nitrilotriacetic acid (NTA), ethylenediaminetetraacetic acid (EDTA), diethylenetriaminepentaacetic acid (DTMPA), alkyl- or alkenylsuccinic acid, soluble silicates or layered silicates (e.g. SKS-6 from Hoechst).

The detergent builders may be subdivided into phosphorus-containing and non-phosphorous-containing types. Examples of phosphorus-containing inorganic alkaline detergent builders include the water-soluble salts, especially alkali metal pyrophosphates, orthophosphates, polyphosphates and phosphonates. Examples  
5 of non-phosphorus-containing inorganic builders include water-soluble alkali metal carbonates, borates and silicates, as well as layered disilicates and the various types of water-insoluble crystalline or amorphous aluminosilicates of which zeolites are the best known representatives.

Examples of suitable organic builders include alkali metal, ammonium or  
10 substituted ammonium salts of succinates, malonates, fatty acid malonates, fatty acid sulphonates, carboxymethoxy succinates, polyacetates, carboxylates, polycarboxylates, aminopolycarboxylates and polyacetyl carboxylates.

The detergent may also be unbuilt, i.e. essentially free of detergent builder.

The detergent may comprise one or more polymers. Examples are  
15 carboxymethylcellulose (CMC; typically in the form of the sodium salt), poly(vinylpyrrolidone) (PVP), polyethyleneglycol (PEG), poly(vinyl alcohol) (PVA), polycarboxylates such as polyacrylates, polymaleates, maleic/acrylic acid copolymers and lauryl methacrylate/acrylic acid copolymers.

The detergent composition may contain bleaching agents of the chlorine/bromine-  
20 type or the oxygen-type. The bleaching agents may be coated or encapsulated. Examples of inorganic chlorine/bromine-type bleaches are lithium, sodium or calcium hypochlorite or hypobromite as well as chlorinated trisodium phosphate. The bleaching system may also comprise a  $H_2O_2$  source such as perborate or percarbonate which may be combined with a peracid-forming bleach activator  
25 such as tetraacetylenediamine (TAED) or nonanoyloxybenzenesulfonate (NOBS).



Examples of organic chlorine/bromine-type bleaches are heterocyclic N-bromo and N-chloro imides such as trichloroisocyanuric, tribromoisocyanuric, dibromoisocyanuric and dichloroisocyanuric acids, and salts thereof with water solubilizing cations such as potassium and sodium. Hydantoin compounds are also suitable. The bleaching system may also comprise peroxyacids of, e.g., the amide, imide, or sulfone type.

In dishwashing detergents the oxygen bleaches are preferred, for example in the form of an inorganic persalt, preferably with a bleach precursor or as a peroxy acid compound. Typical examples of suitable peroxy bleach compounds are alkali metal perborates, both tetrahydrates and monohydrates, alkali metal percarbonates, persilicates and perphosphates. Preferred activator materials are TAED or NOBS.

The enzymes of the detergent composition of the invention may be stabilized using conventional stabilizing agents, e.g. a polyol such as propylene glycol or glycerol, a sugar or sugar alcohol, lactic acid, boric acid, or a boric acid derivative such as, e.g., an aromatic borate ester, and the composition may be formulated as described in, e.g., WO 92/19709 and WO 92/19708. The enzymes of the invention may also be stabilized by adding reversible enzyme inhibitors, e.g., of the protein type (as described in EP 0 544 777 B1) or the boronic acid type.

The detergent may also contain other conventional detergent ingredients such as, e.g., fabric conditioners including clays, deflocculant material, foam boosters/foam depressors (in dishwashing detergents foam depressors), suds suppressors, anti-corrosion agents, soil-suspending agents, anti-soil-redeposition agents, dyes, dehydrating agents, bactericides, optical brighteners, or perfume.

The pH (measured in aqueous solution at use concentration) will usually be neutral or alkaline, e.g. in the range of 7-11.

Particular forms of laundry detergent compositions within the scope of the invention include:

1) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

5	Linear alkylbenzenesulfonate (calculated as acid)	7 - 12%
	Alcohol ethoxysulfate (e.g. C <sub>12-18</sub> alcohol, 1-2 EO) or alkyl sulfate (e.g. C <sub>16-18</sub> )	1 - 4%
	Alcohol ethoxylate (e.g. C <sub>14-15</sub> alcohol, 7 EO)	5 - 9%
10	Sodium carbonate (as Na <sub>2</sub> CO <sub>3</sub> )	14 - 20%
	Soluble silicate (as Na <sub>2</sub> O, 2SiO <sub>2</sub> )	2 - 6%
	Zeolite (as NaAlSiO <sub>4</sub> )	15 - 22%
	Sodium sulfate (as Na <sub>2</sub> SO <sub>4</sub> )	0 - 6%
15	Sodium citrate/citric acid (as C <sub>6</sub> H <sub>5</sub> Na <sub>3</sub> O <sub>7</sub> /C <sub>6</sub> H <sub>8</sub> O <sub>7</sub> )	0 - 15%
	Sodium perborate (as NaBO <sub>3</sub> ·H <sub>2</sub> O)	11 - 18%
	TAED	2 - 6%
	Carboxymethylcellulose	0 - 2%
20	Polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	0 - 3%
	Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
	Minor ingredients (e.g. suds suppressors, perfume, optical brightener, photobleach)	0 - 5%

2) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

	Linear alkylbenzenesulfonate (calculated as acid)	6 - 11%
5	Alcohol ethoxysulfate (e.g. C <sub>12-18</sub> alcohol, 1-2 EO or alkyl sulfate (e.g. C <sub>16-18</sub> ))	1 - 3%
	Alcohol ethoxylate (e.g. C <sub>14-15</sub> alcohol, 7 EO)	5 - 9%
	Sodium carbonate (as Na <sub>2</sub> CO <sub>3</sub> )	15 - 21%
10	Soluble silicate (as Na <sub>2</sub> O, 2SiO <sub>2</sub> )	1 - 4%
	Zeolite (as NaAlSiO <sub>4</sub> )	24 - 34%
	Sodium sulfate (as Na <sub>2</sub> SO <sub>4</sub> )	4 - 10%
	Sodium citrate/citric acid (as C <sub>6</sub> H <sub>5</sub> Na <sub>3</sub> O <sub>7</sub> /C <sub>6</sub> H <sub>8</sub> O <sub>7</sub> )	0 - 15%
15	Carboxymethylcellulose	0 - 2%
	Polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	1 - 6%
	Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
20	Minor ingredients (e.g. suds suppressors, perfume)	0 - 5%

3) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

5	Linear alkylbenzenesulfonate (calculated as acid)	5 - 9%
	Alcohol ethoxylate (e.g. C <sub>12-15</sub> alcohol, 7 EO)	7 - 14%
	Soap as fatty acid (e.g. C <sub>16-22</sub> fatty acid)	1 - 3%
	Sodium carbonate (as Na <sub>2</sub> CO <sub>3</sub> )	10 - 17%
	Soluble silicate (as Na <sub>2</sub> O, 2SiO <sub>2</sub> )	3 - 9%
10	Zeolite (as NaAlSiO <sub>4</sub> )	23 - 33%
	Sodium sulfate (as Na <sub>2</sub> SO <sub>4</sub> )	0 - 4%
	Sodium perborate (as NaBO <sub>3</sub> ·H <sub>2</sub> O)	8 - 16%
	TAED	2 - 8%
	Phosphonate (e.g. EDTMPA)	0 - 1%
15	Carboxymethylcellulose	0 - 2%
	Polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	0 - 3%
	Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
20	Minor ingredients (e.g. suds suppressors, perfume, optical brightener)	0 - 5%

4) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

5	Linear alkylbenzenesulfonate (calculated as acid)	8	- 12%
	Alcohol ethoxylate (e.g. C <sub>12-15</sub> alcohol, 7 EO)	10	- 25%
	Sodium carbonate (as Na <sub>2</sub> CO <sub>3</sub> )	14	- 22%
	Soluble silicate (as Na <sub>2</sub> O, 2SiO <sub>2</sub> )	1	- 5%
	Zeolite (as NaAlSiO <sub>4</sub> )	25	- 35%
10	Sodium sulfate (as Na <sub>2</sub> SO <sub>4</sub> )	0	- 10%
	Carboxymethylcellulose	0	- 2%
	Polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	1	- 3%
	Enzymes (calculated as pure enzyme protein)	0.0001	- 0.1%
15	Minor ingredients (e.g. suds suppressors, perfume)	0	- 5%

5) An aqueous liquid detergent composition comprising

20	Linear alkylbenzenesulfonate (calculated as acid)	15	- 21%
	Alcohol ethoxylate (e.g. C <sub>12-15</sub> alcohol, 7 EO or C <sub>12-15</sub> alcohol, 5 EO)	12	- 18%
	Soap as fatty acid (e.g. oleic acid)	3	- 13%
	Alkenylsuccinic acid (C <sub>12-14</sub> )	0	- 13%
	Aminoethanol	8	- 18%
25	Citric acid	2	- 8%
	Phosphonate	0	- 3%
	Polymers (e.g. PVP, PEG)	0	- 3%
	Borate (as B <sub>4</sub> O <sub>7</sub> )	0	- 2%
30	Ethanol	0	- 3%
	Propylene glycol	8	- 14%

Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. dispersants, suds suppressors, perfume, optical brightener)	0 - 5%

5 6) An aqueous structured liquid detergent composition comprising

10	Linear alkylbenzenesulfonate (calculated as acid)	15 - 21%
	Alcohol ethoxylate (e.g. C <sub>12-15</sub> alcohol, 7 EO, or C <sub>12-15</sub> alcohol, 5 EO)	3 - 9%
	Soap as fatty acid (e.g. oleic acid)	3 - 10%
	Zeolite (as NaAlSiO <sub>4</sub> )	14 - 22%
15	Potassium citrate	9 - 18%
	Borate (as B <sub>4</sub> O <sub>7</sub> )	0 - 2%
	Carboxymethylcellulose	0 - 2%
	Polymers (e.g. PEG, PVP)	0 - 3%
20	Anchoring polymers such as, e.g., lauryl methacrylate/acrylic acid copolymer; molar ratio 25:1; MW 3800	0 - 3%
	Glycerol	0 - 5%
	Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
	Minor ingredients (e.g. dispersants, suds suppressors, perfume, optical brighteners)	0 - 5%

25 7) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

30	Fatty alcohol sulfate	5 - 10%
	Ethoxylated fatty acid monoethanolamide	3 - 9%
	Soap as fatty acid	0 - 3%
	Sodium carbonate (as Na <sub>2</sub> CO <sub>3</sub> )	5 - 10%
	Soluble silicate (as Na <sub>2</sub> O,2SiO <sub>2</sub> )	1 - 4%

	Zeolite (as NaAlSiO <sub>4</sub> )	20	- 40%
	Sodium sulfate (as Na <sub>2</sub> SO <sub>4</sub> )	2	- 8%
	Sodium perborate (as NaBO <sub>3</sub> ·H <sub>2</sub> O)	12	- 18%
	TAED	2	- 7%
5	Polymers (e.g. maleic/acrylic acid copolymer, PEG)	1	- 5%
	Enzymes (calculated as pure enzyme protein)	0.0001	- 0.1%
10	Minor ingredients (e.g. optical brightener, suds suppressors, perfume)	0	- 5%

8) A detergent composition formulated as a granulate comprising

	Linear alkylbenzenesulfonate (calculated as acid)	8	- 14%
	Ethoxylated fatty acid monoethanolamide	5	- 11%
15	Soap as fatty acid	0	- 3%
	Sodium carbonate (as Na <sub>2</sub> CO <sub>3</sub> )	4	- 10%
	Soluble silicate (as Na <sub>2</sub> O, 2SiO <sub>2</sub> )	1	- 4%
	Zeolite (as NaAlSiO <sub>4</sub> )	30	- 50%
	Sodium sulfate (as Na <sub>2</sub> SO <sub>4</sub> )	3	- 11%
20	Sodium citrate (as C <sub>6</sub> H <sub>5</sub> Na <sub>3</sub> O <sub>7</sub> )	5	- 12%
	Polymers (e.g. PVP, maleic/acrylic acid copolymer, PEG)	1	- 5%
	Enzymes (calculated as pure enzyme protein)	0.0001	- 0.1%
25	Minor ingredients (e.g. suds suppressors, perfume)	0	- 5%

9) A detergent composition formulated as a granulate comprising

	Linear alkylbenzenesulfonate (calculated as acid)	6	- 12%
30	Nonionic surfactant	1	- 4%
	Soap as fatty acid	2	- 6%

5	Sodium carbonate (as $\text{Na}_2\text{CO}_3$ )	14	- 22%
	Zeolite (as $\text{NaAlSiO}_4$ )	18	- 32%
	Sodium sulfate (as $\text{Na}_2\text{SO}_4$ )	5	- 20%
	Sodium citrate (as $\text{C}_6\text{H}_5\text{Na}_3\text{O}_7$ )	3	- 8%
	Sodium perborate (as $\text{NaBO}_3 \cdot \text{H}_2\text{O}$ )	4	- 9%
10	Bleach activator (e.g. NOBS or TAED)	1	- 5%
	Carboxymethylcellulose	0	- 2%
	Polymers (e.g. polycarboxylate or PEG)	1	- 5%
	Enzymes (calculated as pure enzyme protein)	0.0001	- 0.1%
	Minor ingredients (e.g. optical brightener, perfume)	0	- 5%

## 10) An aqueous liquid detergent composition comprising

15	Linear alkylbenzenesulfonate (calculated as acid)	15	- 23%
	Alcohol ethoxysulfate (e.g. $\text{C}_{12-15}$ alcohol, 2-3 EO)	8	- 15%
	Alcohol ethoxylate (e.g. $\text{C}_{12-15}$ alcohol, 7 EO, or $\text{C}_{12-15}$ alcohol, 5 EO)	3	- 9%
20	Soap as fatty acid (e.g. lauric acid)	0	- 3%
	Aminoethanol	1	- 5%
	Sodium citrate	5	- 10%
25	Hydrotrope (e.g. sodium toluenesulfonate)	2	- 6%
	Borate (as $\text{B}_4\text{O}_7$ )	0	- 2%
	Carboxymethylcellulose	0	- 1%
30	Ethanol	1	- 3%
	Propylene glycol	2	- 5%
	Enzymes (calculated as pure enzyme protein)	0.0001	- 0.1%
	Minor ingredients (e.g. polymers, dispersants, perfume, optical brighteners)	0	- 5%



## 11) An aqueous liquid detergent composition comprising

	Linear alkylbenzenesulfonate (calculated as acid)	20	- 32%
5	Alcohol ethoxylate (e.g. C <sub>12-15</sub> alcohol, 7 EO, or C <sub>12-15</sub> alcohol, 5 EO)	6	- 12%
	Aminoethanol	2	- 6%
	Citric acid	8	- 14%
	Borate (as B <sub>4</sub> O <sub>7</sub> )	1	- 3%
10	Polymer (e.g. maleic/acrylic acid copolymer, anchoring polymer such as, e.g., lauryl methacrylate/acrylic acid copolymer)	0	- 3%
	Glycerol	3	- 8%
	Enzymes (calculated as pure enzyme protein)	0.0001	- 0.1%
15	Minor ingredients (e.g. hydrotropes, dispersants, perfume, optical brighteners)	0	- 5%

## 12) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

20	Anionic surfactant (linear alkylbenzenesulfonate, alkyl sulfate, alpha-olefinsulfonate, alpha-sulfo fatty acid methyl esters, alkanesulfonates, soap)	25	- 40%
	Nonionic surfactant (e.g. alcohol ethoxylate)	1	- 10%
25	Sodium carbonate (as Na <sub>2</sub> CO <sub>3</sub> )	8	- 25%
	Soluble silicates (as Na <sub>2</sub> O, 2SiO <sub>2</sub> )	5	- 15%
	Sodium sulfate (as Na <sub>2</sub> SO <sub>4</sub> )	0	- 5%
	Zeolite (as NaAlSiO <sub>4</sub> )	15	- 28%
	Sodium perborate (as NaBO <sub>3</sub> ·4H <sub>2</sub> O)	0	- 20%
30	Bleach activator (TAED or NOBS)	0	- 5%
	Enzymes (calculated as pure enzyme protein)	0.0001	- 0.1%
	Minor ingredients (e.g. perfume, optical brighteners)	0	- 3%

13) Detergent formulations as described in 1) - 12) wherein all or part of the linear alkylbenzenesulfonate is replaced by (C<sub>12</sub>-C<sub>18</sub>) alkyl sulfate.

14) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

5	(C <sub>12</sub> -C <sub>18</sub> ) alkyl sulfate	9	- 15%
	Alcohol ethoxylate	3	- 6%
	Polyhydroxy alkyl fatty acid amide	1	- 5%
	Zeolite (as NaAlSiO <sub>4</sub> )	10	- 20%
	Layered disilicate (e.g. SK56 from Hoechst)	10	- 20%
10	Sodium carbonate (as Na <sub>2</sub> CO <sub>3</sub> )	3	- 12%
	Soluble silicate (as Na <sub>2</sub> O, 2SiO <sub>2</sub> )	0	- 6%
	Sodium citrate	4	- 8%
	Sodium percarbonate	13	- 22%
	TAED	3	- 8%
15	Polymers (e.g. polycarboxylates and PVP)	0	- 5%
	Enzymes (calculated as pure enzyme protein)	0.0001	- 0.1%
	Minor ingredients (e.g. optical brightener, photo bleach, perfume, suds suppressors)	0	- 5%

20 15) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

25	(C <sub>12</sub> -C <sub>18</sub> ) alkyl sulfate	4	- 8%
	Alcohol ethoxylate	11	- 15%
	Soap	1	- 4%
	Zeolite MAP or zeolite A	35	- 45%
	Sodium carbonate (as Na <sub>2</sub> CO <sub>3</sub> )	2	- 8%
	Soluble silicate (as Na <sub>2</sub> O, 2SiO <sub>2</sub> )	0	- 4%

	Sodium percarbonate	13 - 22%
	TAED	1 - 8%
	Carboxymethyl cellulose	0 - 3%
	Polymers (e.g. polycarboxylates and PVP)	0 - 3%
5	Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
	Minor ingredients (e.g. optical brightener, phosphonate, perfume)	0 - 3%

16) Detergent formulations as described in 1) - 15) which contain a stabilized or  
 10 encapsulated peracid, either as an additional component or as a substitute for  
 already specified bleach systems.

17) Detergent compositions as described in 1), 3), 7), 9) and 12) wherein  
 perborate is replaced by percarbonate.

18) Detergent compositions as described in 1), 3), 7), 9), 12), 14) and 15) which  
 15 additionally contain a manganese catalyst. The manganese catalyst may, e.g., be  
 one of the compounds described in "Efficient manganese catalysts for low-  
 temperature bleaching", Nature 369, 1994, pp. 637-639.

19) Detergent composition formulated as a nonaqueous detergent liquid  
 comprising a liquid nonionic surfactant such as, e.g., linear alkoxylated primary  
 20 alcohol, a builder system (e.g. phosphate), enzyme and alkali. The detergent may  
 also comprise anionic surfactant and/or a bleach system.

Particular forms of dishwashing detergent compositions within the scope of the  
 invention include:

## 1) POWDER AUTOMATIC DISHWASHING COMPOSITION

	Nonionic surfactant	0.4 - 2.5%
	Sodium metasilicate	0 - 20%
	Sodium disilicate	3 - 20%
5	Sodium triphosphate	20 - 40%
	Sodium carbonate	0 - 20%
	Sodium perborate	2 - 9%
	Tetraacetylenediamine (TAED)	1 - 4%
	Sodium sulphate	5 - 33%
10	Enzymes	0.0001 - 0.1%

## 2) POWDER AUTOMATIC DISHWASHING COMPOSITION

5	Nonionic surfactant (e.g. alcohol ethoxylate)	1 - 2%
	Sodium disilicate	2 - 30%
	Sodium carbonate	10 - 50%
	Sodium phosphonate	0 - 5%
	Trisodium citrate dihydrate	9 - 30%
10	Nitrilotrisodium acetate (NTA)	0 - 20%
	Sodium perborate monohydrate	5 - 10%
	Tetraacetythylenediamine (TAED)	1 - 2%
	Polyacrylate polymer (e.g. maleic acid/acrylic acid copolymer)	6 - 25%
	Enzymes	0.0001 - 0.1%
15	Perfume	0.1 - 0.5%
	Water	5 - 10

## 3) POWDER AUTOMATIC DISHWASHING COMPOSITION

20	Nonionic surfactant	0.5 - 2.0%
	Sodium disilicate	25 - 40%
	Sodium citrate	30 - 55%
	Sodium carbonate	0 - 29%
	Sodium bicarbonate	0 - 20%
25	Sodium perborate monohydrate	0 - 15%
	Tetraacetythylenediamine (TAED)	0 - 6%
	Maleic acid/acrylic acid copolymer	0 - 5%
	Clay	1 - 3%
	Poly(amino acids)	0 - 20%
	Sodium polyacrylate	0 - 8%
	Enzymes	0.0001 - 0.1%

## 4) POWDER AUTOMATIC DISHWASHING COMPOSITION

5	Nonionic surfactant	1 - 2%
	Zeolite MAP	15 - 42%
	Sodium disilicate	30 - 34%
	Sodium citrate	0 - 12%
	Sodium carbonate	0 - 20%
10	Sodium perborate monohydrate	7 - 15%
	Tetraacetythylenediamine (TAED)	0 - 3%
	Polymer	0 - 4%
	Maleic acid/acrylic acid copolymer	0 - 5%
	Organic phosphonate	0 - 4%
	Clay	1 - 2%
	Enzymes	0.0001 - 0.1%
	Sodium sulphate	Balance

## 15 5) POWDER AUTOMATIC DISHWASHING COMPOSITION

20	Nonionic surfactant	1 - 7%
	Sodium disilicate	18 - 30%
	Trisodium citrate	10 - 24%
	Sodium carbonate	12 - 20%
	Monopersulphate (2 KHSO <sub>5</sub> ·KHSO <sub>4</sub> ·K <sub>2</sub> SO <sub>4</sub> )	15 - 21%
25	Bleach stabilizer	0.1 - 2%
	Maleic acid/acrylic acid copolymer	0 - 6%
	Diethylenetriaminepentaacetate, pentasodium salt	0 - 2.5%
	Enzymes	0.0001 - 0.1%
	Sodium sulphate, water	Balance

# 6) POWDER AND LIQUID DISHWASHING COMPOSITION WITH CLEANING SURFACTANT SYSTEM

	Nonionic surfactant	0 - 1.5%
	Octadecyl dimethylamine N-oxide dihydrate	0 - 5%
5	80:20 wt.C18/C16 blend of octadecyl dimethylamine N-oxide dihydrate and hexadecyldimethyl amine N-oxide dihydrate	0 - 4%
10	70:30 wt.C18/C16 blend of octadecyl bis (hydroxyethyl)amine N-oxide anhydrous and hexadecyl bis (hydroxyethyl)amine N-oxide anhydrous	0 - 5%
	C <sub>13</sub> -C <sub>15</sub> alkyl ethoxysulfate with an average degree of ethoxylation of 3	0 - 10%
15	C <sub>12</sub> -C <sub>15</sub> alkyl ethoxysulfate with an average degree of ethoxylation of 3	0 - 5%
	C <sub>13</sub> -C <sub>15</sub> ethoxylated alcohol with an average degree of ethoxylation of 12	0 - 5%
	A blend of C <sub>12</sub> -C <sub>15</sub> ethoxylated alcohols with an average degree of ethoxylation of 9	0 - 6.5%
20	A blend of C <sub>13</sub> -C <sub>15</sub> ethoxylated alcohols with an average degree of ethoxylation of 30	0 - 4%
	Sodium disilicate	0 - 33%
	Sodium tripolyphosphate	0 - 46%
	Sodium citrate	0 - 28%
25	Citric acid	0 - 29%
	Sodium carbonate	0 - 20%
	Sodium perborate monohydrate	0 - 11.5%
	Tetraacetylenediamine (TAED)	0 - 4%
	Maleic acid/acrylic acid copolymer	0 - 7.5%
30	Sodium sulphate	0 - 12.5%
	Enzymes	0.0001 - 0.1%

## 7) NON-AQUEOUS LIQUID AUTOMATIC DISHWASHING COMPOSITION

5	Liquid nonionic surfactant (e.g. alcohol ethoxylates)	2.0 - 10.0%
	Alkali metal silicate	3.0 - 15.0%
	Alkali metal phosphate	20.0 - 40.0%
	Liquid carrier selected from higher glycols, polyglycols, polyoxides, glycolethers	25.0 - 45.0%
	Stabilizer (e.g. a partial ester of phosphoric acid and a C <sub>16</sub> -C <sub>18</sub> alkanol)	0.5 - 7.0%
10	Foam suppressor (e.g. silicone)	0 - 1.5%
	Enzymes	0.0001 - 0.1%

## 8) NON-AQUEOUS LIQUID DISHWASHING COMPOSITION

15	Liquid nonionic surfactant (e.g. alcohol ethoxylates)	2.0 - 10.0%
	Sodium silicate	3.0 - 15.0%
	Alkali metal carbonate	7.0 - 20.0%
	Sodium citrate	0.0 - 1.5%
20	Stabilizing system (e.g. mixtures of finely divided silicone and low molecular weight dialkyl polyglycol ethers)	0.5 - 7.0%
	Low molecule weight polyacrylate polymer	5.0 - 15.0%
	Clay gel thickener (e.g. bentonite)	0.0 - 10.0%
	Hydroxypropyl cellulose polymer	0.0 - 0.6%
	Enzymes	0.0001 - 0.1%
25	Liquid carrier selected from higher glycols, polyglycols, polyoxides and glycol ethers	Balance



## 9) THIXOTROPIC LIQUID AUTOMATIC DISHWASHING COMPOSITION

	C <sub>12</sub> -C <sub>14</sub> fatty acid	0	- 0.5%
	Block co-polymer surfactant	1.5	- 15.0%
	Sodium citrate	0	- 12%
5	Sodium tripolyphosphate	0	- 15%
	Sodium carbonate	0	- 8%
	Aluminium tristearate	0	- 0.1%
	Sodium cumene sulphonate	0	- 1.7%
	Polyacrylate thickener	1.32	- 2.5%
10	Sodium polyacrylate	2.4	- 6.0%
	Boric acid	0	- 4.0%
	Sodium formate	0	- 0.45%
	Calcium formate	0	- 0.2%
	Sodium n-decyldiphenyl oxide disulphonate	0	- 4.0%
15	Monoethanol amine (MEA)	0	- 1.86%
	Sodium hydroxide (50%)	1.9	- 9.3%
	1,2-Propanediol	0	- 9.4%
	Enzymes	0.0001	- 0.1%
	Suds suppressor, dye, perfumes, water	Balance	

## 20 10) LIQUID AUTOMATIC DISHWASHING COMPOSITION

	Alcohol ethoxylate	0	- 20%
	Fatty acid ester sulphonate	0	- 30%
	Sodium dodecyl sulphate	0	- 20%
	Alkyl polyglycoside	0	- 21%
25	Oleic acid	0	- 10%
	Sodium disilicate monohydrate	18	- 33%
	Sodium citrate dihydrate	18	- 33%

5	Sodium stearate	0 - 2.5%
	Sodium perborate monohydrate	0 - 13%
	Tetraacetythylenediamine (TAED)	0 - 8%
	Maleic acid/acrylic acid copolymer	4 - 8%
	Enzymes	0.0001 - 0.1%

11) LIQUID AUTOMATIC DISHWASHING COMPOSITION CONTAINING PROTECTED BLEACH PARTICLES

10	Sodium silicate	5 - 10%
	Tetrapotassium pyrophosphate	15 - 25%
	Sodium triphosphate	0 - 2%
	Potassium carbonate	4 - 8%
	Protected bleach particles, e.g. chlorine	5 - 10%
15	Polymeric thickener	0.7 - 1.5%
	Potassium hydroxide	0 - 2%
	Enzymes	0.0001 - 0.1%
	Water	Balance

11) Automatic dishwashing compositions as described in 1), 2), 3), 4), 6) and 10), wherein perborate is replaced by percarbonate.

12) Automatic dishwashing compositions as described in 1) - 6) which additionally contain a manganese catalyst. The manganese catalyst may, e.g., be one of the compounds described in "Efficient manganese catalysts for low-temperature bleaching", Nature 369, 1994, pp. 637-639.

An  $\alpha$ -amylase variant of the invention may be incorporated in concentrations conventionally employed in detergents. It is at present contemplated that, in the detergent composition of the invention, the  $\alpha$ -amylase variant may be added in an amount corresponding to 0.00001-1 mg (calculated as pure enzyme protein) of  $\alpha$ -amylase per liter of wash/dishwash liquor.

### Textile desizing

In the textile processing industry,  $\alpha$ -amylases are traditionally used as auxiliaries in the desizing process to facilitate the removal of starch-containing size which has served as a protective coating on weft yarns during weaving.

- 5 Complete removal of the size coating after weaving is important to ensure optimum results in the subsequent processes, in which the fabric is scoured, bleached and dyed. Enzymatic starch break-down is preferred because it does not involve any harmful effect on the fibre material.

10 In order to reduce processing cost and increase mill throughput, the desizing processing is sometimes combined with the scouring and bleaching steps. In such cases, non-enzymatic auxiliaries such as alkali or oxidation agents are typically used to break down the starch, because traditional  $\alpha$ -amylases are not very compatible with high pH levels and bleaching agents. The non-enzymatic breakdown of the starch size does lead to some fibre damage because of the  
15 rather aggressive chemicals used.

Accordingly, it would be desirable to use  $\alpha$ -amylase enzymes having an improved resistance towards or compatible with oxidation (bleaching) agents at elevated pH, in order to retain the advantages of enzymatic size break down in a time-saving simultaneous desizing/scouring/bleaching process.

- 20 Thus,  $\alpha$ -amylase variants of the invention having improved resistance towards oxidation agents will be useful in desizing processes as described above, in particular for replacement of non-enzymatic alkali or oxidation agents used today.

The present invention is further described with reference to the appended drawing, in which:

Fig. 1 is a restriction map of plasmid pTVB106; and

Fig. 2 is an alignment of the following amino acid sequences:

- 5        1: the amino acid sequence shown in SEQ ID No. 1;
- 2: the amino acid sequence shown in SEQ ID No. 2;
- 3: the amino acid sequence shown in SEQ ID No. 3; and
- 4: the amino acid sequence of a "*Bacillus* sp. #707" amylase as described  
         by Tsukamoto et al. in Biochem. Biophys. Res. Commun. 151 (1988) pp.  
10       25-31.

## EXPERIMENTAL SECTION

Preparation of the parent  $\alpha$ -amylases having the amino acid sequences shown in SEQ ID No. 1 and SEQ ID No. 2, respectively, from *Bacillus* strain NCIB 12512 and NCIB 12513, respectively.

### 5. Fermentation:

Each of the above-mentioned *Bacillus* strains was incubated at 26°C on a rotary shaking table (300 r.p.m.) in 500 ml baffled Erlenmeyer flasks containing 100 ml of BP-X medium + 0.1 M Carbonate buffer pH 9.0.

BP-X medium:

10	Potato starch	100 g
	Ground barley	50 g
	Soybean flour	20 g
	Sodium caseinate	10 g
	Na <sub>2</sub> HPO <sub>4</sub> ·12 H <sub>2</sub> O	9 g
15	Termamyl® 60L *	0.1 g
	Pluronic®	0.1 g

\*) available from Novo Nordisk A/S.

The starch in the medium was liquified by slowly heating the medium from 60°C to 85°C for 30 minutes. After this the temperature of the medium was quickly raised to 95°C for 10 minutes, and the medium was then cooled. Lastly, the medium was sterilized by heating at 121°C for 40 minutes.

### Purification of the $\alpha$ -amylase from strain NCIB 12512

After 5 days of incubation the culture broth was filtered and concentrated using a Filtron™ ultrafiltration module with 3KD membranes and washed with deionized water until the conductivity was 1 mS/cm. The pH was adjusted to pH 5.9 with 10% (v/v) acetic acid. A S-Sepharose FF column was equilibrated in EKV-buffer, pH 5.9. If not otherwise stated, the purification buffer was 100 mM boric acid,

10 mM succinic acid, 2 mM  $\text{CaCl}_2$ , (EKV-buffer) adjusted to the indicated pH with NaOH.

The enzyme solution was applied to the column, the column was washed with EKV-buffer, pH 5.9, and the amylase was eluted with a linear NaCl gradient (0 -> 500 mM NaCl). Amylase-containing fractions were pooled and the pH adjusted to pH 7 with 3% (w/v) NaOH.

A chelate agarose column was loaded with  $\text{Cu}^{2+}$  and equilibrated in the following manner: 50 mM  $\text{CuSO}_4$ , pH 5, was pumped on to the column until the whole column was blue, then excess of  $\text{Cu}^{2+}$  ions were removed by washing the column with 500 mM imidazole, pH 7, and finally the column was equilibrated with EKV-buffer, pH 7. The amylase pool from the S-Sepharose column was applied to the  $\text{Cu}^{2+}$ -loaded chelate agarose column, the column was washed with EKV-buffer, pH 7, and the enzyme was eluted with a linear gradient of imidazole (0 -> 500 mM imidazole). Amylase-containing fractions were pooled and a solution of saturated ammonium sulphate was added to give a final concentration of 1M  $(\text{NH}_4)_2\text{SO}_4$  in the pool.

A phenyl sepharose column was equilibrated in EKV-buffer + 1M  $(\text{NH}_4)_2\text{SO}_4$ , pH 7. The amylase pool from the  $\text{Cu}^{2+}$  column was applied to the hydrophobic interaction column. Binding experiments had shown that the amylase is a rather hydrophobic enzyme, and hence binds tightly to the phenyl column. Protein which did not bind as tightly to the column was washed off the column with EKV-buffer, pH 7. The amylase was step-eluted from the column with EKV-buffer + 25% (v/v) isopropanol. The amylase-containing pool was adjusted to pH 9.5 with 3% (w/v) NaOH and diluted 5 times with deionized water.

A Q-Sepharose HP column was equilibrated in 20 mM Tris-HCl, pH 9.5. The amylase pool from the phenyl sepharose column was applied to the column and the column was washed with 20 mM Tris-HCl, pH 9.5. The amylase was eluted

with a linear gradient of NaCl (0 -> 250 mM NaCl). The amylase peak was adjusted to pH 7 with 10% (v/v) acetic acid.

A  $\text{Cu}^{2+}$ -loaded chelating Sepharose FF column (loaded with  $\text{Cu}^{2+}$  as described for the chelate agarose column) was equilibrated with EKV-buffer, pH 7. The amylase peak from the Q-Sepharose column was applied to the column, and the column was washed thoroughly with EKV-buffer, pH 7. The amylase was eluted with a steep linear gradient of imidazole (0 -> 500 mM imidazole).

The purified amylase was purity checked by SDS-PAGE electrophoresis. The Coomassie-stained gel had only one band.

#### 10 Purification of the $\alpha$ -amylase from strain NCIB 12513

After 5 days of incubation the culture broth was filtered and concentrated using a Filtron<sup>TM</sup> ultrafiltration module with 3KD membranes. The concentrated solution was filtered and saturated to 20% w/w with ammonium sulfate. The solution was then batch adsorbed using a AFFI-T<sup>TM</sup> matrix from Kem-En-Tec A/S, Copenhagen, Denmark. The amylase was eluted using 25% isopropanol in 20 mM Tris, pH 7.5, after washing of the matrix with deionized water. The eluted enzyme was subjected to dialysis (20 mM Tris, pH 8.5) and a stepwise batch adsorption on Q-Sepharose FF for colour removal was carried out.

A chelate agarose column was loaded with  $\text{Cu}^{2+}$  and equilibrated in the following manner: 50 mM  $\text{CuSO}_4$ , pH 5 was pumped on to the column until the whole column was blue, then excess of  $\text{Cu}^{2+}$  ions was removed by washing the column with 500 mM imidazole, pH 7, and finally the column was equilibrated with 50 mM borate buffer, pH 7.

In spite of the low pI of this amylase (5.8), it was not bound to the Q-Sepharose FF at pH 8.5.

The run-through from the Q-Sepharose FF column was applied on the  $\text{Cu}^{2+}$ -chelating agarose and eluted using 250 mM imidazole, 20 mM Tris, pH 7.0 and the eluted column was dialysed against 50 mM borate buffer, pH 7.0. The pH was adjusted to pH 9.5, and the dialysed solution was bound on a Q-Sepharose HP and eluted over 10 columns using a linear gradient from 0 -> 250 mM NaCl. Amylase-containing fractions were pooled and a solution of saturated ammonium sulphate was added to give a final concentration of 20% w/w, whereafter the fractions were applied on a phenyl Sepharose column. The column was washed using deionized water and eluted using 25% isopropanol in 50 mM borate buffer, pH 7.0.

The purified amylase was purity checked by SDS-PAGE electrophoresis. The Coomassie-stained gel had only one band.

#### Physical/chemical properties of the parent $\alpha$ -amylases

As already indicated, the  $\alpha$ -amylases from strain NCIB 12512 and NCIB 12513 have the amino acid sequences shown in SEQ ID No. 1 and SEQ ID No. 2, respectively. Amino acid sequencing of the two  $\alpha$ -amylases was carried out using standard methods [see, e.g., Findley and Geisow (Eds.), Protein Sequencing - A Practical Approach, IRL Press (1989)].

Some other properties of the two  $\alpha$ -amylases (prepared and purified as described above) are as follows:

SEQ ID No. 1: pI about 8.8-9.0 (determined by isoelectric focusing on LKB Ampholine™ PAG plates); molecular weight approximately 55 kD (determined by SDS-PAGE).

SEQ ID No. 2: pI about 5.8 (determined by isoelectric focusing on LKB Ampholine™ PAG plates); molecular weight approximately 55 kD (determined by SDS-PAGE).



#### Determination of $\alpha$ -amylase activity

$\alpha$ -Amylase activity is determined by a method employing Phadebas® tablets as substrate. Phadebas tablets (Phadebas® Amylase Test, supplied by Pharmacia Diagnostic) contain a cross-linked insoluble blue-coloured starch polymer which  
5 has been mixed with bovine serum albumin and a buffer substance and tabletted.

For the determination of every single measurement one tablet is suspended in a tube containing 5 ml 50 mM Britton-Robinson buffer (50 mM acetic acid, 50 mM phosphoric acid, 50 mM boric acid, 0.1 mM  $\text{CaCl}_2$ , pH adjusted to the value of interest with NaOH). The test is performed in a water bath at the temperature of  
10 interest. The  $\alpha$ -amylase to be tested is diluted in x ml of 50 mM Britton-Robinson buffer. 1 ml of this  $\alpha$ -amylase solution is added to the 5 ml 50 mM Britton-Robinson buffer. The starch is hydrolysed by the  $\alpha$ -amylase giving soluble blue fragments. The absorbance of the resulting blue solution, measured spectrophotometrically at 620 nm, is a function of the  $\alpha$ -amylase activity.

15 It is important that the measured 620 nm absorbance after 15 minutes of incubation (testing time) is in the range of 0.2 to 2.0 absorbance units at 620 nm. In this absorbance range there is linearity between activity and absorbance (Lambert-Beer law). The dilution of the enzyme must therefore be adjusted to fit this criterion.

20 Under a specified set of conditions (temp., pH, reaction time, buffer conditions) 1 mg of a given  $\alpha$ -amylase will hydrolyse a certain amount of substrate and a blue colour will be produced. The colour intensity is measured at 620 nm. The measured absorbance is directly proportional to the specific activity (activity/mg of pure  $\alpha$ -amylase protein) of the  $\alpha$ -amylase in question under the given set of  
25 conditions. Thus testing different  $\alpha$ -amylases of interest (including a reference  $\alpha$ -amylase, in this case the parent  $\alpha$ -amylase in question) under identical conditions, the specific activity of each of the  $\alpha$ -amylases at a given temperature and at a given pH can be compared directly, and the ratio of the specific activity of each

of the  $\alpha$ -amylases of interest relative to the specific activity of the reference  $\alpha$ -amylase can be determined.

#### Mini dishwashing assay

The following mini dishwashing assay was used: A suspension of starchy material was boiled and cooled to 20°C. The cooled starch suspension was applied on small, individually identified glass plates (approx. 2 x 2 cm) and dried at a temperature in the range of 60-140°C in a drying cabinet. The individual plates were then weighed. For assay purposes, a solution of standard European-type automatic dishwashing detergent (5 g/l) having a temperature of 55°C was prepared. The detergent was allowed a dissolution time of 1 minute, after which the  $\alpha$ -amylase in question was added to the detergent solution (contained in a beaker equipped with magnetic stirring) so as to give an enzyme concentration of 0.5 mg/ml. At the same time, the weighed glass plates, held in small supporting clamps, were immersed in a substantially vertical position in the  $\alpha$ -amylase/detergent solution, which was then stirred for 15 minutes at 55°C. The glass plates were then removed from the  $\alpha$ -amylase/detergent solution, rinsed with distilled water, dried at 60°C in a drying cabinet and re-weighed. The performance of the  $\alpha$ -amylase in question [expressed as an index relative to a chosen reference  $\alpha$ -amylase (index 100) - in the present case the parent  $\alpha$ -amylase having the amino acid sequence shown in SEQ ID No. 1] was then determined from the difference in weight of the glass plates before and after treatment, as follows:

$$\text{Index} = \frac{\text{weight loss for plate treated with } \alpha\text{-amylase}}{\text{weight loss for plate treated with reference}} \cdot 100$$

The following examples further illustrate the present invention, and they are not intended to be in any way limiting to the scope of the invention as claimed.

**EXAMPLE 1**Mini dishwashing test of deletion variant T183\* + G184\*

The above-described mini dishwashing test was performed at pH 10.5 with the parent  $\alpha$ -amylase having the amino acid sequence shown in SEQ ID No. 1 and the thermally more stable deletion variant T183\* + G184\*. The test gave the following results:

parent $\alpha$ -amylase	Index: 100
T183* + G184*	Index: 120

The results clearly illustrate the beneficial effect on dishwashing performance which may be achieved using a variant according to the invention having improved thermal stability.

**EXAMPLE 2**Construction of variants of the parent  $\alpha$ -amylase having the amino acid sequence shown in SEQ ID No. 1

15 Description of plasmid pTVB106: The parent  $\alpha$ -amylase having the amino acid sequence shown in SEQ ID No. 1 and variants thereof are expressed from a plasmid-borne gene, SF16, shown in Fig. 1. The plasmid, pTVB106, contains an origin of replication obtained from plasmid pUB110 (Gryczan et al., 1978) and the cat gene conferring resistance towards chloramphenicol. Secretion of the amylase  
20 is aided by the Termamyl™ signal sequence that is fused precisely, i.e. codon No.1 of the mature protein, to the gene encoding the parent  $\alpha$ -amylase having the nucleotide and amino acid sequence (mature protein) shown in SEQ ID No. 4 and SEQ ID No. 1, respectively. The Termamyl promoter initiates transcription of the gene.

Plasmid pTVB106 is similar to pDN1528 (see laid-open Danish patent application No. 1155/94). Some unique restriction sites are indicated on the plasmid map in Fig. 1, i.e. SacII, BamHI, BstEII, AflIII, DraIII, XmaI, SalI and BglII.

Construction of variant M202T: The PCR overlap extension mutagenesis method is used to construct this variant (Higuchi et al., 1988). An approximately 350 bp DNA fragment of pTVB106 is amplified in a PCR reaction A using primers #7113 and mutagenic primer #6778. In a similar PCR reaction B, an approximately 300 bp DNA fragment is amplified using primers Y296 and #6779. The complete DNA fragment spanning the mutation site (M202) from primer #7113 to primer Y296 is amplified in PCR C using these primers and purified DNA fragments from reactions A and B.

PCR C DNA is digested with restriction endonucleases BstEII and AflIII, and the 480 bp fragment is ligated with plasmid pTVB106 digested with the same enzymes and transformed into a low-protease and low-amylase *Bacillus subtilis* strain (e.g. strain SHA273 mentioned in WO 92/11357).

Construction of variants T183\* + G184\* and R181\* + G182\*: The PCR overlap extension mutagenesis method is used to construct these variants (Higuchi et al., 1988). The mutagenic oligoneucleotides are synthesized using a mixture (equal parts) of C and G in one position; two different mutations can therefore be constructed by this procedure. An approximately 300 bp DNA fragment of pTVB106 is amplified in a PCR reaction A using primers #7113 and mutagenic primer #7449. In a similar PCR reaction B, an approximately 400 bp DNA fragment is amplified using primers Y296 and #3811. The complete DNA fragment spanning the mutation site (amino acids 181-184) from primer #7113 to primer Y296 is amplified in PCR C using these primers and purified DNA fragments from reactions A and B.

PCR C DNA is digested with restriction endonucleases BstEII and AflIII and the 480 bp fragment is ligated with plasmid pTVB106 digested with the same

enzymes and transformed into a low-protease and low-amylase *B. subtilis* strain (e.g. strain SHA273 mentioned in WO 92/11357). Sequencing of plasmid DNA from these transformants identifies the two correct mutations: i.e. R181\* + G182\* and T183\* + G184\*.

5 Primers:

#7113:

5' GCT GCG GTG ACC TCT TTA AAA AAT AAC GGC 3'

Y296:

5' CC ACC GCT ATT AGA TGC ATT GTA C 3'

10 #6779

5' CTT ACG TAT GCA GAC GTC GAT ATG GAT CAC CC 3'

#6778

5' G ATC CAT ATC GAC GTC TGC ATA CGT AAG ATA GTC 3'

#3811

15 5' TT A(C/G)G GGC AAG GCC TGG GAC TGG 3'

#7449

5'C CCA GGC CTT GCC C(C/G)T AAA TTT ATA TAT TTT GTT TTG 3'

**EXAMPLE 3**Determination of oxidation stability of variant M202T relative to the parent  $\alpha$ -amylase having the amino sequence shown in SEQ ID No. 1

The enzyme variant to be tested (in this case M202T; see, e.g., Example 2) was  
5 purified to homogeneity in a manner analogous to that described above for the  
parent  $\alpha$ -amylase having the amino acid sequence shown in SEQ ID No. 1. The  
buffer was changed to 50mM Britton-Robinson buffer (50 mM acetic acid, 50  
mM phosphoric acid, 50 mM boric acid, 0.1 mM  $\text{CaCl}_2$ , pH adjusted to the value  
of interest with NaOH), pH 9,0. Hydrogen peroxide was added to the enzyme  
10 solution to give a final concentration of 200mM  $\text{H}_2\text{O}_2$ , and the solution was then  
incubated at 40°C in a water bath.

After 2, 4, 6, 10, 15 and 30 minutes of incubation the residual  $\alpha$ -amylase activity  
was measured using the Phadebas assay described above. The residual activity  
in the samples was measured using 50mM Britton-Robinson buffer, pH 7,3, at  
15 37°C (see Novo analytical publication AF207-1/1, available on request from Novo  
Nordisk A/S). The decline in activity was measured relative to a reference sample  
of the same enzyme at 0 minutes which was not incubated with hydrogen  
peroxide (100% activity).

The percentage of initial activity as a function of time is shown in the table below  
20 for the parent enzyme and for the variant in question.

Time in min.	SEQ ID No.1	variant M202T
0	100	100
4	57	90
6	42	67
10	31	52
15	14	37
30	2	24

The variant M202T clearly exhibits significantly improved stability towards oxidation relative to the parent  $\alpha$ -amylase in question.

10 Determination of thermal stability of the deletion variant T183\* + G184\* relative to the parent  $\alpha$ -amylase having the amino sequence shown in SEQ ID No. 1

The enzyme variant to be tested (in this case T183\* + G184\*; see, e.g., Example 2) was purified to homogeneity in a manner analogous to that described above for the parent  $\alpha$ -amylase having the amino acid sequence shown in SEQ ID No. 1. The buffer was changed to 50mM Glycine buffer, 0,1mM CaCl<sub>2</sub>, pH 10,5. The enzyme solution was then incubated in a water bath at 55°C.

After 5, 10, 15 and 30 minutes of incubation the residual activity was measured using the Phadebas assay described above. The residual activity in the samples was measured using 50mM Glycine buffer, pH 7,3, 37°C (see Novo analytical publication AF207-1/1, available on request from Novo Nordisk A/S). The decline in activity was measured relative to a reference sample of the same enzyme at 0 minutes which was not incubated (100% activity).

min.	SEQ ID No. 1	T183* + G184*
0	100%	100%
5	77%	89%
10	68%	89%
15	70%	85%
30	37%	86%

The variant T183\* + G184\* clearly exhibits significantly improved stability at 55°C relative to the parent  $\alpha$ -amylase in question.



**REFERENCES CITED IN THE SPECIFICATION**

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## SEQUENCE DESCRIPTION: SEQ ID No. 1

His His Asn Gly Thr Asn Gly Thr Met Met Gln Tyr Phe Glu Trp Tyr  
1  
Leu Pro Asn Asp Gly Asn His Trp Asn Arg Leu Arg Asp Asp Ala Ala  
5  
Asn Leu Lys Ser Lys Gly Ile Thr Ala Val Trp Ile Pro Pro Ala Trp  
Lys Gly Thr Ser Gln Asn Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr  
10 Asp Leu Gly Glu Phe Asn Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly  
Thr Arg Asn Gln Leu Gln Ala Ala Val Thr Ser Leu Lys Asn Asn Gly  
Ile Gln Val Tyr Gly Asp Val Val Met Asn His Lys Gly Gly Ala Asp  
15 Gly Thr Glu Ile Val Asn Ala Val Glu Val Asn Arg Ser Asn Arg Asn  
Gln Glu Thr Ser Gly Glu Tyr Ala Ile Glu Ala Trp Thr Lys Phe Asp  
20 Phe Pro Gly Arg Gly Asn Asn His Ser Ser Phe Lys Trp Arg Trp Tyr  
His Phe Asp Gly Thr Asp Trp Asp Gln Ser Arg Gln Leu Gln Asn Lys  
Ile Tyr Lys Phe Arg Gly Thr Gly Lys Ala Trp Asp Trp Glu Val Asp  
25 Thr Glu Asn Gly Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Val Asp Met  
Asp His Pro Glu Val Ile His Glu Leu Arg Asn Trp Gly Val Trp Tyr  
30 Thr Asn Thr Leu Asn Leu Asp Gly Phe Arg Ile Asp Ala Val Lys His  
Ile Lys Tyr Ser Phe Thr Arg Asp Trp Leu Thr His Val Arg Asn Thr

Thr Gly Lys Pro Met Phe Ala Val Ala Glu Phe Trp Lys Asn Asp Leu  
Gly Ala Ile Glu Asn Tyr Leu Asn Lys Thr Ser Trp Asn His Ser Val  
5 Phe Asp Val Pro Leu His Tyr Asn Leu Tyr Asn Ala Ser Asn Ser Gly  
Gly Tyr Tyr Asp Met Arg Asn Ile Leu Asn Gly Ser Val Val Gln Lys  
10 His Pro Thr His Ala Val Thr Phe Val Asp Asn His Asp Ser Gln Pro  
Gly Glu Ala Leu Glu Ser Phe Val Gln Gln Trp Phe Lys Pro Leu Ala  
Tyr Ala Leu Val Leu Thr Arg Glu Gln Gly Tyr Pro Ser Val Phe Tyr  
15 Gly Asp Tyr Tyr Gly Ile Pro Thr His Gly Val Pro Ala Met Lys Ser  
Lys Ile Asp Pro Leu Leu Gln Ala Arg Gln Thr Phe Ala Tyr Gly Thr  
20 Gln His Asp Tyr Phe Asp His His Asp Ile Ile Gly Trp Thr Arg Glu  
Gly Asn Ser Ser His Pro Asn Ser Gly Leu Ala Thr Ile Met Ser Asp  
Gly Pro Gly Gly Asn Lys Trp Met Tyr Val Gly Lys Asn Lys Ala Gly  
25 Gln Val Trp Arg Asp Ile Thr Gly Asn Arg Thr Gly Thr Val Thr Ile  
Asn Ala Asp Gly Trp Gly Asn Phe Ser Val Asn Gly Gly Ser Val Ser  
30 Val Trp Val Lys Gln  
485

## SEQUENCE DESCRIPTION: SEQ ID No. 2

His His Asn Gly Thr Asn Gly Thr Met Met Gln Tyr Phe Glu Trp His  
1  
Leu Pro Asn Asp Gly Asn His Trp Asn Arg Leu Arg Asp Asp Ala Ser  
5  
Asn Leu Arg Asn Arg Gly Ile Thr Ala Ile Trp Ile Pro Pro Ala Trp  
Lys Gly Thr Ser Gln Asn Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr  
10 Asp Leu Gly Glu Phe Asn Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly  
Thr Arg Ser Gln Leu Glu Ser Ala Ile His Ala Leu Lys Asn Asn Gly  
Val Gln Val Tyr Gly Asp Val Val Met Asn His Lys Gly Gly Ala Asp  
15 Ala Thr Glu Asn Val Leu Ala Val Glu Val Asn Pro Asn Asn Arg Asn  
Gln Glu Ile Ser Gly Asp Tyr Thr Ile Glu Ala Trp Thr Lys Phe Asp  
20 Phe Pro Gly Arg Gly Asn Thr Tyr Ser Asp Phe Lys Trp Arg Trp Tyr  
His Phe Asp Gly Val Asp Trp Asp Gln Ser Arg Gln Phe Gln Asn Arg  
Ile Tyr Lys Phe Arg Gly Asp Gly Lys Ala Trp Asp Trp Glu Val Asp  
25 Ser Glu Asn Gly Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Val Asp Met  
Asp His Pro Glu Val Val Asn Glu Leu Arg Arg Trp Gly Glu Trp Tyr  
0  
30 Thr Asn Thr Leu Asn Leu Asp Gly Phe Arg Ile Asp Ala Val Lys His  
Ile Lys Tyr Ser Phe Thr Arg Asp Trp Leu Thr His Val Arg Asn Ala

Thr Gly Lys Glu Met Phe Ala Val Ala Glu Phe Trp Lys Asn Asp Leu  
Gly Ala Leu Glu Asn Tyr Leu Asn Lys Thr Asn Trp Asn His Ser Val  
5 Phe Asp Val Pro Leu His Tyr Asn Leu Tyr Asn Ala Ser Asn Ser Gly  
Gly Asn Tyr Asp Met Ala Lys Leu Leu Asn Gly Thr Val Val Gln Lys  
10 His Pro Met His Ala Val Thr Phe Val Asp Asn His Asp Ser Gln Pro  
Gly Glu Ser Leu Glu Ser Phe Val Gln Glu Trp Phe Lys Pro Leu Ala  
Tyr Ala Leu Ile Leu Thr Arg Glu Gln Gly Tyr Pro Ser Val Phe Tyr  
15 Gly Asp Tyr Tyr Gly Ile Pro Thr His Ser Val Pro Ala Met Lys Ala  
Lys Ile Asp Pro Ile Leu Glu Ala Arg Gln Asn Phe Ala Tyr Gly Thr  
20 Gln His Asp Tyr Phe Asp His His Asn Ile Ile Gly Trp Thr Arg Glu  
Gly Asn Thr Thr His Pro Asn Ser Gly Leu Ala Thr Ile Met Ser Asp  
Gly Pro Gly Gly Glu Lys Trp Met Tyr Val Gly Gln Asn Lys Ala Gly  
25 Gln Val Trp His Asp Ile Thr Gly Asn Lys Pro Gly Thr Val Thr Ile  
Asn Ala Asp Gly Trp Ala Asn Phe Ser Val Asn Gly Gly Ser Val Ser  
30 Ile Trp Val Lys Arg  
485

## SEQUENCE DESCRIPTION: SEQ ID No. 3

1	AAPFNGTMMQ YFEWYLPDDG TLWTKVANE	NNLSSLGITA LWLPPAYKGT	
	SRSDVGYG VY DLYDLGEFNQ KGAVRTKYGT	KAQYLQAIQA AEAAGMQVYA	100
	DVVFDHKGGA DGT EWDAVE VNPSDRNQEI	SGTYQIQAWT KFD FPGRGNT	
	YSSF KWRWYH FDGVDWDESR KLSRIYKFRG	IGKAWDWEVD TENGNYDYLM	200
	YADLDMDHPE VVTELKSWGK WYVNTTNIDG	FRLDAVKHIK FSFFPDWLS	
	VRSQTGKPLF TVGEYWSYDI NKLHNYIMKT	NGTMSLFDAP LHNKFYTASK	300
	SGGT FDMRTL MTNTLMKDQP TLAVTFVDNH	DTEPGQALQS WVDPWFKPLA	
	YAFILTRQEG YPCVFYGDYY GIPQYNIPSL	KSKIDPLLIA RRDYAYGTQH	400
	DYLDHSDIIG WTREGVTEKP GSGLAALITD	GPGGSKWMYV GKQHAGKV FY	
	DLTG NRSDTV TINS DGWGEF KVNGGSVSVW	VPRKTTVSTI AWSITTRPWT	500
	DEFVRWTEPR LVAW		514

## SEQUENCE DESCRIPTION: SEQ ID No. 4

CATCATAATG GAACAAATGG TACTATGATG CAATATTTTCG AATGGTATTT GCCAAATGAC 60  
GGGAATCATT GGAACAGGTT GAGGGATGAC GCAGCTAACT TAAAGAGTAA AGGGATAACA 120  
GCTGTATGGA TCCCACCTGC ATGGAAGGGG ACTTCCCAGA ATGATGTAGG TTATGGAGCC 180  
5 TATGATTTAT ATGATCTTGG AGAGTTTAAC CAGAAGGGGA CGGTTTCGTAC AAAATATGGA 240  
ACACGCAACC AGCTACAGGC TCGGGTGACC TCTTTAAAAA ATAACGGCAT TCAGGTATAT 300  
GGTGATGTCG TCATGAATCA TAAAGGTGGA GCAGATGGTA CGGAAATTGT AAATGCGGTA 360  
GAAGTGAATC GGAGCAACCG AAACCAGGAA ACCTCAGGAG AGTATGCAAT AGAAGCGTGG 420  
ACAAAGTTTG ATTTTCCTGG AAGAGGAAAT AACCATTCCA GCTTTAAGTG GCGCTGGTAT 480  
10 CATTTTGATG GGACAGATTG GGATCAGTCA CGCCAGCTTC AAAACAAAAT ATATAAATTC 540  
AGGGGAACAG GCAAGGCCTG GGAAGTGGAA GTCGATACAG AGAATGGCAA CTATGACTAT 600  
CTTATGTATG CAGACGTGGA TATGGATCAC CCAGAAGTAA TACATGAACT TAGAAACTGG 660  
GGAGTGTGGT ATACGAATAC ACTGAACCTT GATGGATTGA GAATAGATGC AGTGAAACAT 720  
ATAAAATATA GCTTTACGAG AGATTGGCTT ACACATGTGC GTAACACCAC AGGTAAACCA 780  
15 ATGTTTGCAG TGGCTGAGTT TTGGAAAAAT GACCTTGGTG CAATTGAAAA CTATTTGAAT 840  
AAAACAAGTT GGAATCACTC GGTGTTTGAT GTTCCTCTCC ACTATAATTT GTACAATGCA 900  
TCTAATAGCG GTGGTTATTA TGATATGAGA AATATTTTAA ATGGTTCTGT GGTGCAAAAA 960  
CATCCAACAC ATGCCGTTAC TTTTGTTGAT AACCATGATT CTCAGCCCGG GGAAGCATTG 1020  
GAATCCTTTG TTCAACAATG GTTTAAACCA CTTGCATATG CATTGGTTCT GACAAGGGAA 1080  
20 CAAGGTTATC CTTCCGTATT TTATGGGGAT TACTACGGTA TCCCAACCCA TGGTGTTCCG 1140  
GCTATGAAAT CTAAAATAGA CCCTCTTCTG CAGGCACGTC AACTTTTTGC CTATGGTACG 1200  
CAGCATGATT ACTTTGATCA TCATGATATT ATCGGTTGGA CAAGAGAGGG AAATAGCTCC 1260  
CATCCAAATT CAGGCCTTGC CACCATTATG TCAGATGGTC CAGGTGGTAA CAAATGGATG 1320  
TATGTGGGGA AAAATAAAGC GGGACAAGTT TGGAGAGATA TTACCGGAAA TAGGACAGGC 1380  
25 ACCGTCACAA TTAATGCAGA CGGATGGGGT AATTTCTCTG TTAATGGAGG GTCCGTTTCG 1440  
GTTTGGGTGA AGCAA 1455



## SEQUENCE DESCRIPTION: SEQ ID No. 5

CATCATAATG GGACAAATGG GACGATGATG CAATACTTTG AATGGCACTT GCCTAATGAT 60  
GGGAATCACT GGAATAGATT AAGAGATGAT GCTAGTAATC TAAGAAATAG AGGTATAACC 120  
GCTATTTGGA TTCCGCCTGC CTGGAAAGGG ACTTCGCAAA ATGATGTGGG GTATGGAGCC 180  
5 TATGATCTTT ATGATTTAGG GGAATTTAAT CAAAAGGGGA CGGTTCGTAC TAAGTATGGG 240  
ACACGTAGTC AATTGGAGTC TGCCATCCAT GCTTTAAAGA ATAATGGCGT TCAAGTTTAT 300  
GGGGATGTAG TGATGAACCA TAAAGGAGGA GCTGATGCTA CAGAAAACGT TCTTGCTGTC 360  
GAGGTGAATC CAAATAACCG GAATCAAGAA ATATCTGGGG ACTACACAAT TGAGGCTTGG 420  
ACTAAGTTTG ATTTTCCAGG GAGGGGTAAT ACATACTCAG ACTTTAAATG GCGTTGGTAT 480  
10 CATTTGATG GTGTAGATTG GGATCAATCA CGACAATTCC AAAATCGTAT CTACAAATTC 540  
CGAGGTGATG GTAAGGCATG GGATTGGGAA GTAGATTCGG AAAATGGAAA TTATGATTAT 600  
TTAATGTATG CAGATGTAGA TATGGATCAT CCGGAGGTAG TAAATGAGCT TAGAAGATGG 660  
GGAGAATGGT ATACAAATAC ATTAAATCTT GATGGATTTA GGATCGATGC GGTGAAGCAT 720  
ATTAAATATA GCTTTACACG TGATTGGTTG ACCCATGTAA GAAACGCAAC GGGAAAAGAA 780  
15 ATGTTTGCTG TTGCTGAATT TTGGAAAAAT GATTTAGGTG CCTTGGAGAA CTATTTAAAT 840  
AAAACAAACT GGAATCATTC TGTCTTTGAT GTCCCCCTTC ATTATAATCT TTATAACGCG 900  
TCAAATAGTG GAGGCAACTA TGACATGGCA AAACCTCTTA ATGGAACGGT TGTTCAAAAG 960  
CATCCAATGC ATGCCGTAAC TTTTGTGGAT AATCACGATT CTCAACCTGG GGAATCATT 1020  
GAATCATTTG TACAAGAATG GTTTAAGCCA CTTGCTTATG CGCTTATTTT AACAAGAGAA 1080  
20 CAAGGCTATC CCTCTGTCTT CTATGGTGAC TACTATGGAA TTCCAACACA TAGTGTCCCA 1140  
GCAATGAAAG CCAAGATTGA TCCAATCTTA GAGGCGCGTC AAAATTTTGC ATATGGAACA 1200  
CAACATGATT ATTTTGACCA TCATAATATA ATCGGATGGA CACGTGAAGG AAATACCACG 1260  
CATCCCAATT CAGGACTTGC GACTATCATG TCGGATGGGC CAGGGGGAGA GAAATGGATG 1320  
TACGTAGGGC AAAATAAAGC AGGTCAAGTT TGGCATGACA TAACTGGAAA TAAACCAGGA 1380  
25 ACAGTTACGA TCAATGCAGA TGGATGGGCT AATTTTTCAG TAAATGGAGG ATCTGTTTCC 1440  
ATTGGGTGA AACGA

1455

## SEQUENCE DESCRIPTION: SEQ ID No. 6

1	GCCGCACCGT	TTAACGGCAC	CATGATGCAG	TATTTTGAAT	GGTACTTGCC	
	GGATGATGGC	ACGTTATGGA	CCAAAGTGGC	CAATGAAGCC	AACAACCTAT	100
	CCAGCCTTGG	CATCACCGCT	CTTTGGCTGC	CGCCCGCTTA	CAAAGGAACA	
	AGCCGCAGCG	ACGTAGGGTA	CGGAGTATAC	GACTTGTATG	ACCTCGGCGA	200
	ATTCAATCAA	AAAGGGACCG	TCCGCACAAA	ATACGGAACA	AAAGCTCAAT	
	ATCTTCAAGC	CATTCAAGCC	GCCCACGCCG	CTGGAATGCA	AGTGTACGCC	300
	GATGTCGTGT	TCGACCATAA	AGGCGGCGCT	GACGGCACGG	AATGGGTGGA	
	CGCCGTGCAA	GTCAATCCGT	CCGACCGCAA	CCAAGAAATC	TCGGGCACCT	400
	ATCAAATCCA	AGCATGGACG	AAATTTGATT	TTCCCGGGCG	GGGCAACACC	
	TACTCCAGCT	TTAAGTGGCG	CTGGTACCAT	TTTGACGGCG	TTGATTGGGA	500
	CGAAAGCCGA	AAATTGAGCC	GCATTTACAA	ATTCCGCGGC	ATCGGCAAAG	
	CGTGGGATTG	GGAAGTAGAC	ACGGAAAACG	GAAACTATGA	CTACTTAATG	600
	TATGCCGACC	TTGATATGGA	TCATCCCGAA	GTCGTGACCG	AGCTGAAAAA	
	CTGGGGGAAA	TGGTATGTCA	ACACAACGAA	CATTGATGGG	TTCCGGCTTG	700
	ATGCCGTCAA	GCATATTAAG	TTCAGTTTTT	TTCCTGATTG	GTTGTCGTAT	
	GTGCGTTCTC	AGACTGGCAA	GCCGCTATTT	ACCGTCGGGG	AATATTGGAG	800
	CTATGACATC	AACAAGTTGC	ACAATTACAT	TACGAAAACA	GACGGAACGA	
	TGTCTTTGTT	TGATGCCCCG	TTACACAACA	AATTTTATAC	CGCTTCCAAA	900
	TCAGGGGGCG	CATTTGATAT	GCGCACGTTA	ATGACCAATA	CTCTCATGAA	
	AGATCAACCG	ACATTGGCCG	TCACCTTCGT	TGATAATCAT	GACACCGAAC	1000
	CCGGCCAAGC	GCTGCAGTCA	TGGGTCGACC	CATGGTTCAA	ACCGTTGGCT	
	TACGCCTTTA	TTCTAACTCG	GCAGGAAGGA	TACCCGTGCG	TCTTTTATGG	1100
	TGACTATTAT	GGCATTCCAC	AATATAACAT	TCCTTCGCTG	AAAAGCAAAA	
	TCGATCCGCT	CCTCATCGCG	CGCAGGGATT	ATGCTTACGG	AACGCAACAT	1200
	GATTATCTTG	ATCACTCCGA	CATCATCGGG	TGGACAAGGG	AAGGGGGCAC	
	TGAAAAACCA	GGATCCGGAC	TGGCCGCACT	GATCACCGAT	GGGCCGGGAG	1300
	GAAGCAAATG	GATGTACGTT	GGCAAACAAC	ACGCTGGAAG	AGTGTTCTAT	
	GACCTTACCG	GCAACCGGAG	TGACACCGTC	ACCATCAACA	GTGATGGATG	1400
	GGGGGAATTC	AAAGTCAATG	GCGGTTCCGT	TTCGGTTTGG	GTTCCCTAGAA	
	AAACGACCGT	TTCTACCATC	GCTCGGCCGA	TCACAACCCG	ACCGTGGACT	1500
	GGTGAATTCG	TCCGTTGGAC	CGAACCACGG	TTGGTGGCAT	GGCCTTGA	1548

## CLAIMS

1. A variant of a parent  $\alpha$ -amylase, which parent  $\alpha$ -amylase (i) has one of the amino acid sequences shown in SEQ ID No. 1, SEQ ID No. 2 and SEQ ID No. 3, respectively; or (ii) displays at least 80% homology with one or more of said amino acid sequences, and/or displays immunological cross-reactivity with an antibody raised against an  $\alpha$ -amylase having one of said amino acid sequences, and/or is encoded by a DNA sequence which hybridizes with the same probe as a DNA sequence encoding an  $\alpha$ -amylase having one of said amino acid sequences; in which variant:
- 5 (a) at least one amino acid residue of said parent  $\alpha$ -amylase has been deleted; and/or
- (b) at least one amino acid residue of said parent  $\alpha$ -amylase has been replaced by a different amino acid residue; and/or
- (c) at least one amino acid residue has been inserted relative to said parent
- 15  $\alpha$ -amylase;
- said variant having  $\alpha$ -amylase activity and exhibiting at least one of the following properties relative to said parent  $\alpha$ -amylase: increased thermostability; increased stability towards oxidation; reduced  $\text{Ca}^{2+}$  dependency.
2. A variant according to claim 1, wherein at least one oxidizable amino acid
- 20 residue of said parent  $\alpha$ -amylase has been deleted or has been replaced by a different amino acid residue which is less susceptible to oxidation than said oxidizable amino acid residue.
3. A variant according to claim 2, wherein said oxidizable amino acid residue is selected from the group consisting of methionine, tryptophan, cysteine and
- 25 tyrosine.
4. A variant according to claim 2 or 3, wherein said oxidizable amino acid residue is a methionine which is, or which is equivalent to, M9, M10, M105, M202,

M208, M261, M309, M382, M430 or M440 of the amino acid sequence shown in SEQ ID No. 1.

5. A variant according to claim 4, comprising a methionine substitution which is, or which is equivalent to, one of the following substitutions in the amino acid sequence shown in SEQ ID No. 1: M9L; M10L; M202L,T,F,I,V; M261L; M309L; M382L; M430L; M440L.

6. A variant according to any one of claims 3-5, wherein a said methionine residue has been replaced by threonine.

7. A variant according to any one of the preceding claims, wherein at least one amino acid which is, or which is equivalent to, F180, R181, G182, T183, G184 or K185 of the amino acid sequence shown in SEQ ID No. 1 has been deleted.

8. A variant according to claim 7, wherein the deleted amino acids are, or are equivalent to, any two of said amino acid residues.

9. A variant according to claim 8, wherein the deletions are, or are equivalent to, R181\* + G182\*; or T183\* + G184\*.

10. A variant according to any one of the preceding claims, comprising an amino acid substitution which is, or which is equivalent to, one of the following substitutions in the amino acid sequence shown in SEQ ID No. 1: P260E; R124P; M105F,I,L,V; M208F,W,Y; L217I; V206I,L,F.

11. A variant according to any one of the preceding claims, comprising an amino acid substitution which is, or which is equivalent to, one of the following substitutions in the amino acid sequence shown in SEQ ID No. 1: Y243F; K108R; K179R; K239R; K242R; K269R; D163N; D188N; D192N; D199N; D205N; D207N; D209N; E190Q; E194Q; N106D.

12. A DNA construct comprising a DNA sequence encoding an  $\alpha$ -amylase variant according to any one of claims 1-11.
13. A recombinant expression vector which carries a DNA construct according to claim 12.
- 5 14. A cell which is transformed with a DNA construct according to claim 12 or a vector according to claim 13.
15. A cell according to claim 14, which is a microorganism.
16. A cell according to claim 15, which is a bacterium or a fungus.
17. A cell according to claim 16, which is a grampositive bacterium such as  
10 *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus lentus*, *Bacillus brevis*, *Bacillus stearothermophilus*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus lautus*, *Bacillus thuringiensis* or *Streptomyces lividans* or *Streptomyces murinus*, or a gramnegative bacterium such as *E.coli*.
- 15 18. A method of producing an  $\alpha$ -amylase variant according to any one of claims 1-11, wherein a cell according to any one of claims 14-17 is cultured under conditions conducive to the production of the  $\alpha$ -amylase variant, and the  $\alpha$ -amylase variant is subsequently recovered from the culture.
19. Use of an  $\alpha$ -amylase variant according to any one of claims 1-11 for washing  
20 and/or dishwashing.
20. A detergent additive comprising an  $\alpha$ -amylase variant according to any one of claims 1-11, optionally in the form of a non-dusting granulate, stabilised liquid or protected enzyme.

21. A detergent additive according to claim 20, comprising 0.02-200 mg of enzyme protein per gram of the additive.
22. A detergent additive according to claim 20 or 21, which additionally comprises another enzyme such as a protease, a lipase, a peroxidase, another  
5 amylolytic enzyme and/or a cellulase.
23. A detergent composition comprising an  $\alpha$ -amylase variant according to any one of claims 1-11.
24. A detergent composition according to claim 23, which additionally comprises another enzyme such as a protease, a lipase, a peroxidase, another amylolytic  
10 enzyme and/or a cellulase.
25. A manual or automatic dishwashing detergent composition comprising an  $\alpha$ -amylase variant according to any one of claims 1-11.
26. A dishwashing detergent composition according to claim 25, which additionally comprises another enzyme such as a protease, a lipase, a peroxidase,  
15 another amylolytic enzyme and/or a cellulase.
27. A manual or automatic laundry washing composition comprising an  $\alpha$ -amylase variant according to any one of claims 1-11.
28. A laundry washing composition according to claim 27, which additionally comprises another enzyme such as a protease, a lipase, a peroxidase, an  
20 amylolytic enzyme and/or a cellulase.
29. Use of an  $\alpha$ -amylase variant according to any one of claims 1-11 for textile desizing.

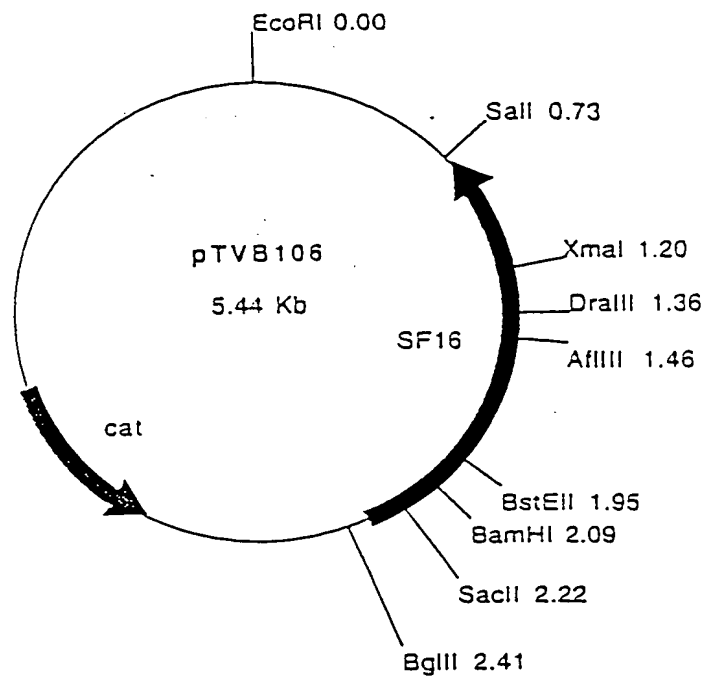


Fig. 1

SEQUENCE	10	20	30	40	50	60	Res		
1	HHNGTNGTMMQYFEWYLPNDGNHWNRLRDDAANLKSXGITA	VWIPP	AWKGT	SQNDV	GYGA		60		
3	-AAPFNGTMMQYFEWYLPDDGTLWTKVANEANNLSSLGIT	ALWLP	PAYKG	TSRSD	VG	YGV	59		
2	HHNGTNGTMMQYFEWYLPNDGNHWNRLRDDASNLRNRGIT	AIWIP	PAWKGT	SQNDV	GYGA		60		
4	HHNGTNGTMMQYFEWYLPNDGNHWNRLNSDASNLSXGITA	VWIP	PAWKGA	SQNDV	GYGA		60		
SEQUENCE	70	80	90	100	110	120	Res		
1	YDLYDLGEFNNQKGVTRTKYGTNRQLQAAVTSLKNNGIQVY	GDVVMNHKGGAD	GTEIV	NAV			120		
3	YDLYDLGEFNNQKGVTRTKYGTAKQYLQAIQAAHAAGMQVY	ADVVF	DHKGGA	DGTEW	VD	AV	119		
2	YDLYDLGEFNNQKGVTRTKYGTNRSQLESAIAHLKNNGVQVY	GDVVMNHKGGAD	ATENV	IAV			120		
4	YDLYDLGEFNNQKGVTRTKYGTNRSQLQAAVTSLKNNGIQVY	GDVVMNHKGGAD	ATEM	VRAV			120		
SEQUENCE	130	140	150	160	170	180	Res		
1	EVNRSNRNQETSGEYAIEAWTKFDFPGRGNNHSSFKWRWYH	FDGTD	WDQSR	QLQNK	IYKF		180		
3	EVNPSDRNQEISGTYQIQAWTKFDFPGRGNTYSSFKWRWYH	FDGVD	WDESR	KLS-RIYKF			178		
2	EVNPNNRNQEIISGDYTI	EAWTKFDFPGRGNTY	SDFKWRWYH	FDGVD	WDQSR	QFQNR	IYKF	180	
4	EVNPNNRNQEVITGEYTI	EAWTRFDFPGRGNTH	SFKWRWYH	FDGVD	WDQSR	RLNNR	IYKF	180	
SEQUENCE	190	200	210	220	230	240	Res		
1	RGTGKAWDWEVDTENGNYDYL	MYADV	MDHPE	VIHEL	RNWGV	WYTN	TLNLDGFRIDAVKH	240	
3	RGIGKAWDWEVDTENGNYDYL	MYADLD	MDHPE	VVTEL	KNWGK	WYVNT	TNIDGFRIDAVKH	238	
2	RGDGKAWDWEVDS	ENGNYDYL	MYADV	MDHPE	VVNEL	RRWGE	WYTN	TLNLDGFRIDAVKH	240
4	RGHGKAWDWEVDTENGNYDYL	MYADID	MDHPE	VVNEL	RNWGV	WYTN	TLGLDGFRIDAVKH	240	
SEQUENCE	250	260	270	280	290	300	Res		
1	IKYSFTRDWLTHVRNTTGKPMFAVAEFWKNDLGA	IENYL	NKTSWNH	SAF	DVPLH	YNLYNA	300		
3	IKFSFFPDWLSYVRSQTGKPLFTVGEYWSYD	INKLH	NYITK	TGTM	SLF	DAPLH	NKFYTA	298	
2	IKYSFTRDWLTHVRNATGKEMFAVAEFWKNDLGA	IENYL	NKTNWNH	SVF	DVPLH	YNLYNA	300		
4	IKYSFTRDWLTHVRSATGKNMFAVAEFWKNDLGA	IENYL	QKTNWNH	SVF	DVPLH	YNLYNA	300		
SEQUENCE	310	320	330	340	350	360	Res		
1	SNSGGYYDMRNILNGSVVQKHPTHAVTFVDNHDS	QPG	EAL	ESFVQ	QWFK	PLAYALV	LTRI	360	
3	SKSGGA	FD	MRT	LT	MNT	LMKD	QPT	LAVTFVDNHDSQPGALQSWDPWFKPLAYAFILTRQ	358
2	SNSGGNYDMAKLLNGT	VVQKH	PMH	AVTFVDNHDS	QPG	SLESFVQ	EWFKPLAYALITRE	360	
4	SKSGGNYDMPNIFNGT	VVQRH	PSH	AVTFVDNHDS	QPE	EAL	ESFVEE	WFKPLAYALTITRE	360
SEQUENCE	370	380	390	400	410	420	Res		
1	QGYPSV	FYGDY	YGIP	THGVP	AMKSK	IDP	LLQARQTFAYGTQHDYFDHHDIIIGWTREGNSS	420	
3	EGYPCV	FYGDY	YGIP	QYNIP	SLKSK	IDP	LLIARRDYAYGTQHDYLDHSDIIIGWTREGGTE	418	
2	QGYPSV	FYGDY	YGIP	THSVP	AMKAK	IDP	ILEARQNFAYGTQHDYFDHHDIIIGWTREGNTT	420	
4	QGYPSV	FYGDY	YGIP	THGVP	AMRSK	IDP	ILEARQKYAYGKQNDYLDHHDIIIGWTREGNTA	420	
SEQUENCE	430	440	450	460	470	480	Res		
1	HPNSGLATIMS	DGPGG	NKWMY	VGNK	AGQV	WDITGN	RRTGTVTINADGWGNFNVNGGSVS	480	
3	KPGSGLAALIT	DGPGG	NKWMY	VGKQ	HAGK	VFYDLTGN	RS	DTVTINSDGWGEFKNVNGGSVS	478
2	HPNSGLATIMS	DGPGG	KNWMY	VGNK	AGQV	WDITGN	KPGT	VTINADGWANFNVNGGSVS	480
4	HPNSGLATIMS	DGAGG	SKWMY	VGNK	AGQV	WDITGN	RRTGTVTINADGWGNFNVNGGSVS	480	
SEQUENCE	490	500	510	520	530	540	Res		
1	VWVKQ						485		
3	VWVPRKTTVSTIAR	PITTR	PWTGE	FVR	WTE	PRLVAW	514		
2	IWVKR						485		
4	IWVNK						485		

FIG. 2



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